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(54) Spectroscopic method

(57) The invention provides a method of identifying an analyte in a sample, said method comprising:

i) treating said sample with a deuterating agent;
ii) directing monochromatic light at said sample;
iii) detecting Raman light signal from said sample;
iv) and comparing said Raman light signal with a standard whereby to identify said analyte.

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Description

This invention relates to a spectroscopic method, in particular a method for analyte identification using Raman spectroscopy.

Recent outbreaks of pathogenic E. Coli 0157:H7 poisoning in humans from the ingestion of tainted meats has created great interest in establishing rational and scientifically tested criteria for approving meat and poultry for human consumption. At the present time, most methods in use providing the selectivity and sensitivity necessary to detect the presence of pathogenic bacteria in or on beef or poultry require 24-48 hours. Test that take this long are based upon classical bacteriological techniques that require samples taken from beef or poultry to be grown up in cultures for bacteriological identification and quantification.

A new instrument that utilizes the polymerase chain reaction (PCR) for bacterial identification is being marketed by Dupont under the trade name Ribo-Printer. This instrument/method takes at least 8 hours to obtain a result.

In "Biological Particle Identification Apparatus" invented by Gary C. Salzman, Charles T. Gregg, W. Kevin Grace, Richard D. Hiebert, U.S. Patent Number 4,884,886 awarded in December 5, 1989, the inventors show that multiparameter light scattering measurements can be used to identify biological particles, such as bacteria and viruses, in pure homogeneous solutions. This requires that each of the particles to be identified be purified prior to analysis. This technique cannot work with heterogeneous samples and is therefore unsuitable for quickly identifying and quantifying bacterial pathogens in beef menstrua in the slaughterhouse.

I have now found that analytes, e.g. biological contaminants, in a sample may efficiently be identified using a Raman spectroscopic method in which the sample is pretreated with a deuterating agent.

Thus viewed from one aspect the invention provides a method of identifying an analyte, preferably a deuteratable analyte, in a sample, said method comprising:

i) treating said sample with a deuterating agent, e.g. deuterium oxide (D_2O);
ii) directing monochromatic light at said sample;
iii) detecting a Raman light signal from said sample;
iv) and comparing said Raman light signal with a standard whereby to identify said analyte.

The deuterating agent used in the method of the invention may be any agent capable of introducing deuterium atoms into the analyte of interest or a competing analyte, ie. one which in the absence of deuteration is not readily distinguishable in terms of Raman spectrum from the analyte of interest. The deuterating agent preferably is one which allows H-D exchange, e.g. D_2O.

While deuteration is generally preferred, the invention may be performed using alternative hydrogen isotopes, other than of course ^1H. Thus, if desired, tritiation may be effected, and the terms deuterating, deuterated and deuterium in the claims hereof should be understood to include tritiating, tritiated and tritium.

In the method of the invention, identification of the analyte may be effected so as to detect the presence or absence of particular analytes or alternatively and preferably to quantify the concentration of such analytes in the sample.

The sample used in the method of the invention may be of biological or non-biological origin; preferably however it is of biological origin or contains material of biological origin, e.g. it may be a sample containing body fluids, waste, cells or tissues and potentially containing biological contaminants such as bacteria, viruses, yeasts, fungi, prions or inorganic or organic products of such contaminants, or fragments of such contaminants. Thus for example the sample may contain biomolecules, biomolecular assemblies, and inorganic or organic molecules. Such biological contaminants and products or fragments of biological contaminants as well as such biomolecules, biomolecular assemblies, and inorganic or organic molecules may be analytes for the purposes of the method of the invention.

The Raman light signal detected using the method of the invention is preferably detected over a plurality of wavelengths, e.g. as a spectrum, and comparison with a standard using the method of the invention preferably involves comparison with characteristic Raman spectral data for the desired analyte pretreated with a deuterating agent.

More especially preferably the method of the invention is a method comprising the steps of:

deuterating with dideuterium oxide (D_2O) a sample containing one or more analytes, each analyte having exchangeable protons such that said deuteration will cause protons of said analyte to be exchanged with deuterons;
providing a monochromatic light at an excitation frequency onto a sample for producing Raman sample light and rejecting Rayleigh light;
passing said Raman sample light through a depolarizer for producing randomized polarization components of said Raman sample light;
generating a Raman sample spectrum calibrated with respect to an absolute differential Raman cross-section standard in response to said randomized polarization components;
providing said Raman sample spectrum to a spectral analyzer; and

providing the identity of one or more said analytes present in said sample, and/or
providing the quantity (ie. indicating the content or concentration) of one or more said analytes in said sample.

In the method of the invention, the monochromatic light directed at the deuterating agent treated sample especially preferably impinges on a surface of said sample which is not in contact with a container wall, ie. an exposed surface, particularly a surface moving relative to the incident light, e.g. the surface of a stream or more preferably a falling curtain of a liquid sample. In this way, the incident light beam does not cause photodecomposition of the analyte in the sample and hence incorrect identification of the analyte, e.g. a false positive or false negative for the presence of a particular analyte or an incorrect concentration for a particulate analyte.

The use in the method of the invention of a polarization scrambler is also a particularly important facet of the method of the invention as this enables comparison standards to be usable on apparatus other than the apparatus on which they were generated, ie. results from one monochromator configuration become usable in connection with apparatus with other monochromator configurations.

The method of the present invention is thus clearly distinct from the method of Nelson et al. described in US-A-4847198. Nelson et al. claimed that showed that resonance Raman spectra of pure cultures of bacteria exhibit taxonomic identifiers. Nelson et al. by collecting resonance Raman spectra as a function of laser excitation frequency, one could carry out a method of taxonomic identification using the excitation behavior of the Raman spectra of the species in question. The excitation behavior is the behavior of the resonance Raman spectra of the same sample as the excitation frequency is varied. Nelson et al. used a quartz capillary as a sample holder through which the analyte solution was flowed, and upon which the laser excitation beam was impinged. The Raman light can be collected in a geometry from 0 to 90 degrees from the collection optical axis. However, What this method gives with one hand it takes away with the other. The design of the Raman system in the Nelson et al. patent is subject to spectral artifacts due to laser induced photodecomposition of the quartz capillary, as well as denaturation and photodecomposition of the biomolecules at the quartz-liquid interface. This may cause changes in the biomolecules' resonance Raman spectra. Furthermore, Nelson et al. did not use a polarization scrambler at the entrance to the monochromator. The lack of a polarization scrambler can lead to anomalous spectra, because the gratings and mirrors in a monochromator preferentially pass light of a given polarization with higher relative efficiency than another polarization. A grating monochromator therefore exhibits a polarization bias that must be removed if the results from one monochromator configuration are to be compared accurately with those of another. Furthermore, when comparing spectra, or the absolute differential Raman cross-sections of Raman bands obtained at different excitation frequencies, a depolarizer should be employed so that the observed changes in these observables remain unaffected by the polarization bias of the monochromator. Thus, the excitation behavior of resonance Raman spectra and the absolute differential Raman cross-sections of the Raman bands in the spectra are only fully reliable if a depolarizer is used in conjunction with a grating monochromator.

Viewed from a further aspect, the invention provides an apparatus suitable for use in the method of the invention, said apparatus comprising:

i) a reservoir for a sample, preferably a liquid sample;
ii) pump means for creating a continuous flow, preferably a planar flow, of said sample in a light exposure zone in which the flowing sample is not in contact with a container wall;
iii) means for directing a beam of monochromatic light onto the surface of said sample in said light exposure zone;
iv) means for detecting Raman light from said sample in said light exposure zone, said means for detecting being disposed so as substantially not to detect Rayleigh scattered light from said sample; and optionally but preferably
v) depolarization means disposed to depolarize Raman light from said sample before wavelength separation and detection thereof.

The apparatus of the invention conveniently comprises:

a reservoir for said sample;
a pump for continuously drawing said analyte, ie. for drawing said sample from said reservoir;
a planar flow device having a windowless sample stream between a pair of bookends said pair having a first end and a second end such that said analyte is continously flowing from said pump into the first end, between said bookends for being exposed to said monochromatic light, wherein the angle of the normal of said windowless sample stream is between 0 and 90 degrees with respect to the collection optic for rejecting Rayleigh scattering, and exiting said planar flow device.

Also described herein is a novel sample holder suitable for creating a planar, exposed, moving sample surface for monochromatic light to be directed at in the method of the invention. Thus viewed from a yet further aspect the invention provides a sample holder for a spectrometer comprising:

i) a reservoir for a liquid sample;
ii) pump means for pumping said sample from said reservoir to a planar flow device having a downwardly opening horizontally elongate aperture with downwardly extending planar flow edge defining means at the ends thereof; and optionally
iii) funnel means for receiving liquid sample flowing from said aperture and returning said liquid sample to said reservoir.

The method and apparatus of the present invention may thus be used for the taxonomic identification and quantitation of bacteria and/or biomolecules and quantitation and identification of inorganic and/or organic compounds having exchangeable protons. The method may find particular use in the detection of contamination resulting from BSE or scrapie infection in cattle or sheep.

The present invention provides a pathogen detection system which is commercially viable, since it is a) quick on the order of seconds to minutes, b) sensitive, c) able to identify and quantify bacteria in heterogeneous samples consisting of bacteria, blood, fat, muscle, excrement, and other components in beef menstrua or other samples of biological origin, d) able to distinguish between pathogenic and non-pathogenic organisms, and e) inexpensive on a per test basis, and which also f) minimizes the requirements for highly trained personnel and expensive reagents. This is important since in slaughterhouses it is imperative that the number of carcasses tested, as well as the total number of bacteriological tests, be dramatically increased to improve the safety of the meat and poultry supply to the public. The method and apparatus of the invention are particularly suitable since they may be used in the slaughterhouse on the processing line.

Central to the simplicity and efficacy of the present invention is the fact that resonance Raman spectra of two different bacteria (or other contaminants) may be similar when the bacteria sit in an aqueous buffer but differ when the bacteria are deuterated.

According to an embodiment of the present invention, a) a sample of analytes is deuterated with D_2O for facilitating identification and quantitation of analytes in said sample, b) a monochromatic light illuminates a sample of analytes for producing Raman sample light and rejecting Rayleigh light, c) the Raman sample light is passed through a depolarizer for producing randomized polarization components, d) a Raman sample spectrum is generated, calibrated with respect to an absolute differential Raman cross-section standard in response to said randomized polarization components, and e) the Raman sample spectrum is provided to a spectral analyzer for identification and/or quantitation of the analytes.

One advantage of the present invention is that quantitation and identification of biomolecules and bacteria may be accomplished on the order of seconds to minutes rather than hours or days.

Another advantage of this invention is that the sample handling apparatus in this invention minimizes spectral artifacts due to photodecomposition and biomolecular denaturation of the analyte.

A feature of the present invention is that the invention achieves its objects regardless of the parameters of the equipment used to obtain the Raman spectrum.

The invention will now be described further with reference to the accompanying drawings, in which:

Figure 1 is plan view of a resonance Raman spectrometer adapted for use in the method according to the present invention for quantifying and identifying analytes;
Figure 1A is a view of a sample for investigation according to the invention;
Figure 2 is a block diagram of one embodiment of detector electronics as shown in Fig. 1;
Fig. 3 is a block diagram of another embodiment of the detector shown in Fig. 1;
Figure 4 is a schematic diagram of a sample reservoir for holding the analytes;
Figure 4A is close up view of Fig. 4 in the region where monochromatic light hits an analyte-containing sample;
Figure 5 is a representation of the polarization components of the Raman spectrum of acetonitrile at 222 nm excitation, the ordinate scale is normalized to the height of the 932 cm^{-1} band in the measured total intensity spectrum and the abscissa scale is Raman shift in cm^{-1}. The polarization components of acetonitrile shown are (a) the measured perpendicular component spectrum, (b) the measured parallel component spectrum, (c) the calculated corrected perpendicular component spectrum, and (d) the calculated total intensity spectrum of acetonitrile at 222 nm excitation. This representation shows the polarization components used to produce the calculated perpendicular and total intensity spectra;
Figure 6 is a representation of the (a) measured total intensity spectrum and (b) calculated total intensity spectrum of acetonitrile at 222 nm excitation. The ordinate scale for both the measured and calculated total intensity spectra are normalized to the height of their respective 981 cm^{-1} band and the abscissa scale is Raman shift, cm^{-1}. This representation shows the near equivalence of the calculated and measured total intensity spectrum, verifying the methods used to calculate the corrected perpendicular component spectrum;
Figure 7 is a representation of the measured total intensity spectra at 222 nm excitation of (a) Brocothrix thermos-

phacta (BT) in water buffer, (b) Pseudomonas fluorescens (PF) in water buffer, and (c) water buffer. The ordinate is normalized to the height of the 981 cm^{-1} SO_4^{-2} band in each spectrum and the abscissa scale is Raman shift, cm^{-1}. This representation shows the 981 cm^{-1} SO_4^{-2} absolute Raman intensity in each solution and shows that the 1048 cm^{-1} bacterial Raman band of (a) BT and (b) PF is not present in the (c) water buffer;

Figure 8 is a representation of the polarization components of the Raman spectrum of BT at 222 nm excitation. The ordinate is normalized to the height of the 981 cm^{-1} SO_4^{-2} band in the measured total intensity spectrum and the abscissa scale is Raman shift, cm^{-1}. The polarization components of BT shown are (a) the measured total intensity, (b) the measured parallel component, and (c) the calculated corrected perpendicular component. This representation shows that there is negligible intensity in the perpendicular component of the 1048 cm^{-1} band of BT so that the depolarization ratio of the 1048 cm^{-1} band of BT at 222 nm excitation is 0;

Figure 9 is a representation of the resonance Raman spectra of BT at 222 nm excitation subjected to hydrogen-deuterium (H-D) exchange over different time intervals, the ordinate for each spectrum is normalized to the height of the 981 cm^{-1} SO_4^{-2} band and the abscissa scale is Raman shift, cm^{-1}. The spectra shown are (a) BT subjected to 0.3 hours of H-D exchange in D_2O buffer, (b) BT subjected to 9.8 hours of H-D exchange in D_2O buffer, and (c) BT in H_2O buffer. These spectra show the sensitivity of the 1048 cm^{-1} band of BT in H_2O buffer to the effects of hydrogen-deuterium (H-D) exchange; this band disappears after 0.3 hours of H-D exchange and a new band appears at 1044 cm^{-1} after 9.8 hours of H-D exchange;

Figure 10 is a representation of the resonance Raman spectra of PF at 222 nm excitation subjected to hydrogen-deuterium (H-D) exchange over different time intervals. The ordinate for each spectrum is normalized to the height of the 981 cm^{-1} SO_4^{-2} band and the abscissa scale is Raman shift, cm^{-1}. The spectra shown are (a) PF subjected to 0.3 hours of H-D exchange in D_2O buffer, (b) PF subjected to 9.8 hours of H-D exchange in D_2O buffer, and (c) PF in H_2O buffer. These spectra show the sensitivity of the 1048 cm^{-1} band of PF in H_2O buffer to the effects of hydrogen-deuterium (H-D) exchange; this band disappears after 0.3 hours of H-D exchange and a new band appears at 1050 cm^{-1} after 9.8 hours of H-D exchange;

Figure 11 is a representation of the 222 nm excitation resonance Raman spectra of (a) BT and (b) PF that have been subjected to 9.5 hours of (H-D) exchange. The ordinate for each spectrum is normalized to the height of the 981 cm^{-1} SO_4^{-2} band and the abscissa scale is Raman shift, cm^{-1}. This representation shows that the frequency of the bacterial Raman band of BT and PF shift in different directions as a result of H-D exchange;

Figure 12 is a representation of the 222 nm excitation resonance Raman spectra of a beef menstrua-bacteria solution. The ordinate for each spectrum is normalized to the height of the 981 cm^{-1} SO_4^{-2} band and the abscissa scale is Raman shift, cm^{-1}. The topmost spectrum is beef menstrua-bacteria solution in H_2O buffer, below are the spectra acquired 15, 21, 29, 89, 100, 107, and 117 minutes after mixing the beef menstrua with the D_2O buffer. This representation shows the time dependent effects of H-D exchange on the beef menstrua-bacteria solution;

Figure 13 is a representation of the time dependent H-D exchange effects on the heights of some of the Raman bands of the beef menstrua-bacteria solution acquired with 222 nm excitation. The ordinate for the changes in the 1003-7 and 1151 cm^{-1} bands, normalized to their values in H_2O buffer, is shown on the left side of the graph, the ordinate for the height of the 1054 cm^{-1} band is shown on the right side of the graph, while the abscissa is duration of H-D exchange in minutes. This representation shows that the heights of these Raman bands change at different rates;

Figure 14 is a representation of the resonance Raman spectra of (a) BT after 9.8 hours of H-D exchange, (b) PF after 9.8 hours of H-D exchange, and beef menstrua-bacteria after 1.95 hours of H-D exchange at 222 nm excitation. The ordinate is normalized to the height of the 981 cm^{-1} SO_4^{-2} band in each spectrum and the abscissa scale is Raman shift, cm^{-1};

Figure 15 is a graph of depolarization ratio as a function of excitation frequency;

Figure 16 is a depiction of the method used to collect a Raman response profile; and

Figure 17 is a representation of the different regimes of computerized spectral analysis (a) with depolarizer 32 and (b) without depolarizer 32 in the Raman collection optics 35.

In this text, the following definitions are used:

ns	nanosecond, 10^{-9} second
nm	nanometer, 10^{-9} meter
cm^{-1}	wavenumber, 1/cm
O.D.	optical density

BT	Brochothrix thermosphacta American Type Culture Catalogue number 11509
PF	Pseudomonas fluorescens American Type Culture Catalogue number 13525
beef menstrua	the constituents found on the surface of a beef carcass
polarizance	equal to the degree-of-polarization that the polarizer produces in an incident monochromatic beam that is unpolarized

Individual aspects of the method and apparatus of the invention will now be discussed with reference to the accompanying drawings.

ACQUISITION OF THE EXPERIMENTAL RAMAN SPECTRUM USING THE RAMAN SPECTROMETER

Figure 1 shows a Raman spectrometer 1 for using resonance Raman spectroscopy for achieving the identification and quantitation of microorganisms, biomolecules, and organic and inorganic molecules. First, a sample 2 is deuterated with deuterium oxide (D_2O) 3. The sample 2 contains one or more analytes 4 having exchangeable protons such that deuteration causes protons of the analyte to be exchanged with deuterons for facilitating identification of said sample. Hereafter it is understood that the term analytes is interchangeable with bacteria, viruses, fungi, yeasts, prions, biomolecules, biomolecular assemblies, inorganic and organic compounds and vice versa.

In Fig. 1, a laser light source 5 directs monochromatic light 6 at an excitation frequency onto sample 2 contained in a reservoir 10 so as to produce sample Raman light 12 while permitting rejection of Rayleigh light 14.

To alter the extent of deuteration or increase the speed of identification/quantitation of the analytes 4, the reservoir 10 may be responsive to a number of controls. The reservoir 10 includes one or more analytes 4 such as beef menstrua, prions, bacteria, organic and inorganic molecules, proteins, nucleic acids and other biomolecules. Second, the reservoir 10 also contains one or more standards 15 for normalizing/ calibrating analysis of the Raman spectra 12 of analytes 4 in sample 2. This is similar to the use of standards in IR and NMR analysis of organic compounds. The reservoir also may contain one or more denaturants 16 for denaturing the analytes 4. Third, the reservoir 10 may be responsive to temperature control 17. Fourth, an exchange rate control 18 may control the rate of deuterium exchange with analyte protons. This is done in a process analogous to varying pH. (It is an analogous process since pH in a D_2O/water solution has a different connotation from pH in solutions where D_2O is not used).

The sample Raman light 12 is passed through a depolarizer 19 to produce randomized polarization components 20. A Raman sample spectrum 22 is generated - calibrated with respect to an absolute differential Raman cross-section standard 15 in response to said randomized polarization components 20. The Raman sample spectrum 22 is provided through a detector 23 to a spectral analyzer 24 for identification 26 and/or quantitation 28 of the analytes 4. Spectral analyzer 24 includes means 31 for calculating an absolute differential Raman cross-section (ADRCS) and for calculating a depolarization ratio. Means 31 has features for improving the performance of spectral analysis including a local field correction 32, a self-absorption correction 34, a solid angle correction 35 and an instrument efficiency correction 36. The spectral analyzer 24 contains a computer(not shown) and the usual elements for analyzing resonance Raman spectra. Means 31 provides the ADRCS and Raman spectra 22 to a neural network 37 and spectral comparator 38. Both are for identifying and quantitating the analytes 4 in response to the ADRCS or Raman sample spectra 22. The comparator compares the Raman sample spectra 22 to a known Raman sample spectra and thereby determines the identity of the analytes. For example, the Raman sample spectra 22 may be compared to the spectra or sum of spectra for BT and PF. If there is a match, then BT and PF are determined to be the analytes 4. The comparator 38 includes a spectral library with the known resonance Raman spectra of known bacteria, viruses, prions, biomolecules, inorganic and organic compounds and other proton-bearing chemical species, e.g. tens, hundreds, thousands or even millions of such known materials. The neural network 37 achieves the same result - identification and quantitation - using well known training algorithms typical of neural networks.

The Raman spectrometer 1 in Figure 1 is now more fully described: an excimer laser 40 generates 1-2 nanosecond light pulses of 308 nm light at a rate between 50 to 400 Hertz. These pulses are directed into a dye laser 42. For the experiments presented here, the excimer laser 40 pumps dye laser 42 to produce 444 nm laser pulses. The light pulses generated by the excimer in the dye laser 42 are passed through a beta barium borate second harmonic generation (BBO SHG) crystal 44, decreasing the wavelength of the laser light by 1/2. An arrangement of Pellam-Brocca prisms 46 separates the 222 nm light from the 444 nm light. The combination of the excimer laser 40, the tuneable dye laser 42, the second harmonic generation crystal 44, and the Pellam-Brocca prisms 46, is referred to as a laser light source 5. The 222 nm light passes through a Glan-Taylor prism 48 to produce a vertically polarized monochromatic ultraviolet light (VPMUL) beam, the monochromatic light 6. The monochromatic light 6 is then focused by an adjustable laser focusing lens 50 through a 5 mm 90 degree S1-UV prism 52 at a sample 2. The combination of the Glan-Taylor prism

48, the laser focusing lens 50, and the steering prism 52, is referred to as a laser steering optic. The position of the focus of the monochromatic coherent light 6 and sample 2 are adjusted to be coincident with the focus of collection optics 54.

The sample 2 is a flat approximately 2 mm thick planar stream of liquid that is not contained by windows of the reservoir 10. A normal 56 to the stream is oriented 45 degrees with respect to the collection optic axis 57. The angle present in Fig. 1 is shown in separate Fig. 1A. When the monochromatic light 6 impinges upon the windowless sample stream 2, elastically scattered Rayleight laser light 14 is directed away from an f/1 S1-UV biconvex 2" (3.08 cm) diameter collection lens 58, while the sample Raman light 12 (that is scattered omnidirectionally by the sample 2) is collected by the f/1 S1-UV biconvex 2" (3.08 cm) diameter collection lens 58. The sample Raman light 12 is then focused ultimately onto monochromator 59 at an entrance slit with an f/6.5 S1-UV biconvex 2" (3.08 cm) diameter lens 60. This lens 60 f-matches the f-number of the detector 23. A depolarizer 19 is placed between the f/6.5 S1-UV biconvex 2" (3.08 cm) diameter lens 60 and monochromator 59 to scramble the polarization of the sample Raman light 12. This is necessary to remove any monochromator 59/detector 23 bias for a specific polarization of sample Raman light 12. If the depolarizer 19 is not present, excitation dependent Raman spectral intensities of Raman bands in the sample Raman light 12 may show anomalous behavior. Furthermore, this depolarizer 19 is generally a necessary prerequisite for the direct comparison of the data between two or more non-identical monochromators 59. The combination of the collection lens 58, the focusing lens 60, and the depolarizer 19 is referred to as the Raman collection optic 54.

Depolarization ratios can be measured by inserting a stacked plate polarizer 62 between f/1 S1-UV biconvex 2" (3.08 cm) diameter collection lens 58 and f/6.5 S1-UV biconvex 2" (3.08 cm) diameter lens 60, concentric with the optic axis. The stacked plate polarizer 62 consists of 18.4" x 2" (46.7 cm x 3.08 cm) S1-UV Suprasil plates(not shown but known in the art). The parallel and perpendicularly polarized components 20 of the sample Raman light 12 are measured by placing the polarizer 62 along the optic axis, measuring either parallel or perpendicular component, and rotating the polarizer 62 about its long axis 90 degrees to measure the other polarization component. The sample Raman light 12 passes through the depolarizer 19 and enters the monochromator 59 where it is wavelength separated.

The Raman spectrum 22 is provided to spectrum analyzer 24. The spectrum analyzer 24 can take several forms as those skilled in the art of spectral analysis would appreciate.

Control of the detector 23 by detector electronics 63 can take many forms. Two are explained here.

The detector 23 can be an intensified photodiode array (IPDA) detector 72. In Figure 2, the Raman spectrum 22 is focused on an intensified photodiode array (IPDA) detector 72 and transferred to a dedicated computer 74 via interface controller 76. The Raman spectrum 22 falls on the IPDA detector 72 for a selectable time to produce an adequate signal to noise spectrum, then computer controlled dedicated spectral processing software specific for each detector 72 reads the detectors 72, and saves the Raman spectrum 22 in the computer 74. The display of this information in graphical form (spectrum) whose ordinate is proportional to photon flux with an abscissa in wavenumbers, or in a tabular form, is well within the scope of those skilled in the art. The IPDA detector 72 can be operated in gated mode, its exposure synchronized with the laser pulses. For instance, the human operator initiates the experiment at the computer 74 that transmits a signal through an IEEE-488 interface to the controller 76 that in turn activates a high voltage pulser within the IPDA controller 76 that applies high voltage gating pulses to expose the detector to the dispersed Raman light 22 at a frequency and gating width determined by the delay generator 80. The delay generator 80 also sends a triggering pulse to the excimer laser controller 82, the application of the high voltage to IPDA detector 72 is delayed relative to the excimer laser triggering pulse to maximize the Raman signal accumulated at the detector 72. At the conclusion of a predetermined data acquisition time, IPDA detector 72 is read and the spectral information transferred to the computer 74 via the IPDA controller 76.

Figure 3 shows a slightly modified apparatus to collect resonance Raman spectra of bacteria. Detector 23 can be an ICCD detector 90. This Fig. 3 apparatus is modified to use a intensified charge couple device detector (ICCD) 90 to detect the sample Raman light 12. The difference between the apparatus in Figures 2 and 3 are detector control - the electronics used to control and acquire data from ICCD 90. The detector 90 is operated ungated. The human operator initiates the experiment at the computer 74 that transmits a signal to the ICCD controller 91 that controls the application of high voltage potential generated in the ICCD 90 to expose the ICCD 90 to the dispersed sample Raman light 12 for the duration of the data acquisition time. Delay generator 94 sends a triggering pulse to the excimer laser controller 82 to fire the laser at a predetermined repetition rate. At the conclusion of a predetermined data acquisition time the high voltage potential is removed from the ICCD 90, the data is read from the ICCD 90, and the spectral information transferred to the computer 74 via the ICCD controller 91.

RESERVOIR

Fig. 4 shows how monochromatic light 6(Fig. 1) is caused to irradiate sample 2 in a region 93 (the light exposure zone) of windowless sample stream.

The reservoir 10 sample handling apparatus is fully described in Figure 4. Figure 4 shows the how the sample 2

in a windowless sample stream 93 is formed by the planar flow device 95. Liquid is circulated through the planar flow device 95 where it exits through a narrow slit 96, on each side of which are two quartz capillary tubes, tube 97 and tube 98. Both of these tubes 97, 98 serve to form a planar sheet of liquid sample 2 between these tubes 97, 98 as the liquid sample 2 flows down towards the funnel 99. The liquid sample 2 flows through the funnel 99 into the test tube 100. The liquid sample 2 is pumped from the test tube 100 through the Teflon tubing 101 by the pump 102 and is directed into the planar flow device 95 through the device cap 103. With this arrangement of Fig. 4, sample 2 can be recirculated past the monochromatic light 6(Fig. 1) indefinitely. Flowing the sample 2 in front of the monochromatic light 6 reduces the risk of photodecomposition of the sample, as the monochromatic light 6 does not irradiate exactly the same sample molecules with every pulse of monochromatic light 6. The decomposition of the reservoir 10 at the sample container/sample/laser interface is eliminated. If this interface is present, the sample 2 in the region 93 has a propensity to photodecompose at the interface. This eventually produces discolored spots of photodecomposed sample and window material that can give anomalous results, i.e. resonance Raman of photodecomposed sample and/or window material. (Often such windows are made of highly polar substances, such as glass and quartz; material known to bind polar biomolecules such as proteins and nucleic acids. This association of the biomolecules with the window material can denature these molecules, altering their Raman spectra. Elimination of the window material reduces the possibility of spectral artifacts due to biomolecular denaturation.) The reservoir 10 described here also treats the sample 2 gently, unlike similar designs in the literature that employ dye jets. The sample 2 is preferably flowed through the system slowly, e.g. at 10-30 ml/minute. This slow flow rate, gentle handling mode is desirable when working with biological samples so as to reduce the risk of denaturation of biopolymers present in the sample 2. Another positive attribute of the slow flow rate is the smoothness and flatness of the surface of the windowless stream of sample 2. The smoother and flatter the stream of sample 2, the better the Rayleigh rejection. The 45 degree angle of the normal of the windowless sample stream 2 with respect to the collection optic axis efficiently excludes detection of the Rayleigh scattering 14, removing the need present in the Nelson et al. patent (supra) for a cuvette filled with a quinoline solution in front of the entrance slit to reject the Rayleigh light. The quinoline solution can distort the observed spectrum by preferentially absorbing light in one or more regions of the observed Raman spectrum. This effect must be taken into account for quantitative measurements of the Raman cross-section to be reliable.

Fig. 4A illustrates the VPMUL monochromatic light 6 incident on the sample in the region 93 at an angle 45 degrees from the axis 57 of the collection optic for producing a cone of sample Raman light 12 and rejecting Rayleigh light 14.

SAMPLE PREPARATION

Preparation of the sample 2 in Figure 1 is necessary to acquire quantitative resonance Raman spectra and is described below. In addition, calibration of the resonance Raman spectrometer 1 is normally required. Three calibration standards are used: a Raman cross-section standard, a wavelength standard, and a depolarization ratio standard.

BUFFER CONTAINING ABSOLUTE RAMAN CROSS-SECTION STANDARD

Buffer solutions of 0.15 M Na_2SO_4, 0.20 M Na_2HPO_4 and adjusted with HCl to pH 7.25 were prepared. The Na_2SO_4 is the absolute Raman cross-section standard.

PREPARATION OF BEEF MENSTRUA SOLUTIONS

Beef menstrua samples were sponged off of a 500 cm^2 area of a beef carcass, and squeezed into a test tube. The sponge was presoaked with a mixture of TWEEN and NaCl. A mixed culture of bacteria isolated from beef carcasses was added in to the beef menstrua. Thus beef menstrua samples were sponged off of a 500 cm^2 area of a the brisket region of a beef carcass with a microbial sampling sterile sponge moistened with 25 ml of 0.085% (wt/vol) NaCl + 0.05% (vol/vol) Tween 20 adjusted to pH 7.8 in a Whirlpak bag. The solution was expressed from the sponge as it was removed from the bag. The sponge was wiped over the sample area 10 times in both the vertical and horizontal direction, with the collected beef menstrua expressed into a test tube. A mixed culture of bacteria isolated from beef carcasses was added to the beef menstrua.

PREPARATION OF BACTERIAL SOLUTIONS

Pure cultures of Brochothrix thermosphacta ATCC 11509 and Pseudomonas fluorescens ATCC 13525 were carefully swabbed off of slants and suspended in a buffer of 0.15 M Na_2SO_4, 0.20 M Na_2HPO_4 and adjusted with HCl to pH 7.25. These suspensions are referred to as the stock bacterial solutions. The optical densities of these suspensions were measured at 600 nm to determine approximate bacterial concentration in the stock solutions. The stock bacterial cultures and the beef menstrua/bacterial solutions were either mixed with 0.15 M Na_2SO_4, 0.20 M Na_2HPO_4 pH 7.25

in water (H_2O) or 0.15 M Na_2SO_4, 0.20 M Na_2HPO_4 pD 7.25 in heavy water (D_2O). The SO_4^{-2} ion serves as the internal Raman cross-section standard as well as the depolarization ratio standard. Typically, 2 ml of menstrua or bacterial culture stock were mixed with the water or heavy water buffer to bring the total volume up to 12.0 ml.

SPECTRAL CALIBRATION

To acquire quantitative resonance Raman spectra, the Raman spectrometer is calibrated with spectral standards for wavelength and polarizance. The calibration procedures are described below.

WAVELENGTH CALIBRATION

Wavelength calibration was accomplished in the following manner. The spectra were wavelength calibrated by first acquiring the spectra of neat acetonitrile, using the 918 and 1372 cm^{-1} bands to convert from diode or pixel number to wavenumber. The spectra of BT and PF presented here were baseline subtracted. All total intensity measurements were normalized to the 981 cm^{-1} band of the internal standard, SO_4^{-2} ion, setting its peak height to 1.000.

POLARIZANCE CALIBRATION

The depolarization ratio of a Raman band, analyte, standard or other, alluded to in Figure 1 is defined to be:

$$\rho = \frac{I_{perpendicular}}{I_{parallel}}$$

Figure 5 shows the (a) measured perpendicular component spectrum, (b) the measured parallel component spectrum, the (c) calculated perpendicular component spectrum, and (d) the calculated total intensity spectrum of acetonitrile at 222 nm excitation. The depolarization ratio measurements have limited precision because a limited solid angle of Raman light 12 is collected, and light leakage of the parallel component of the Raman light 12 through the polarizer 62 when measuring the perpendicular component. This leakage of one polarization component through the polarizer when measuring the other polarization component is related to the polarizance of the polarizer defined equal to the degree-of-polarization that the polarizer produces in an incident monochromatic beam that is unpolarized. This leakage of the wrong polarization component through the polarizer is approximately corrected for in the following manner. The depolarization ratio, ρ, of the 918 cm^{-1} band of acetonitrile and of the 981 cm^{-1} band of SO_4^{-2} was measured to be no greater than 0.05 between 220 and 300 nm. These depolarization ratios are probably less than 0.05. Since the precision of the depolarization ratio measurements with the stacked plate polarizer is limited, with $\rho = 0.05$ indistinguishable from $\rho = 0$; the value of ρ was set to 0. The residual transmittance of the polarizer was removed from the (a) perpendicular component spectrum by subtracting 0.2 x the (b) parallel component spectrum from perpendicular spectrum to bring the intensity of the 918 cm^{-1} line of acetonitrile in the (c) corrected perpendicular component spectrum to 0. This subtraction process can be verified by adding the (c) corrected perpendicular component to the (a) parallel component to produce a (d) calculated total intensity spectrum. Because of scattering losses in the polarizer, the calculated total intensity spectrum exhibits lower intensity Raman bands than those in the measured total intensity spectrum measured without a depolarizer.

Figure 6 shows (a) the normalized measured total intensity spectrum, normalized with respect to the 981 cm^{-1} band height and (b) calculated total intensity spectrum (setting the height of 981 cm^{-1} band to 1.000). These two spectra nearly overlap. This confirms that the method of calculating the corrected perpendicular component spectrum yields acceptable results. If the results were unacceptable, the spectra would not overlap, when the heights of the 981 cm^{-1} bands were made to be equal to 1, the baselines would be off, or the width of the bands would be off.

CALCULATION OF THE ABSOLUTE RAMAN CROSS-SECTION FROM EXPERIMENTAL DATA

Next in the identification 26 and quantification 28 of bacteria and other analytes 4 (Figure 1) is the calculation of the absolute differential Raman cross-section(ADRCS) and depolarization ratio of Raman bands of the bacteria and other analytes in sample 2. This calculation requires that instrument efficiency 36, solid angle 35, local field 32, and self-absorption 34 corrections need to be considered.

The calculation of the absolute differential Raman cross-section (ADRCS) and the corrections 32, 34, 35, 36 are discussed in this section. The absolute differential Raman cross-sections of the bacteria or any analyte 4 is given by the following equation:

Equation 2

$$\left[\frac{d\sigma}{d\Omega}\right]_{bac} = \left[\frac{d\sigma}{d\Omega}\right]_{std} \frac{I_{bac}}{I_{std}} \frac{E(\bar{\nu}_{laser}-\bar{\nu}_{bac})}{E(\bar{\nu}_{laser}-\bar{\nu}_{std})} \frac{C_{std}}{C_{bac}} \frac{L(bac+std)}{L(std)} \left[\frac{n_{std}}{n_{bac}}\right]^2 (Self\text{-}Abs)$$

where:

$$\left(\frac{d\sigma}{d\Omega}\right)_{bac}$$

is the absolute differential Raman cross section of the bacteria in cm^2/bacteria-steradian

$$\left(\frac{d\sigma}{d\Omega}\right)_{std}$$

is the absolute differential Raman cross section of the internal standard, $SO_4^{-2} = 5.8x10^{-28}$ cm^2/molecule steradian at 222 nm excitation

$$I_{bac} \text{ and } I_{std}$$

are the intensities, heights or areas, of the bacterial or standard Raman vibrational band

$$E(\bar{\nu}_{laser} - \bar{\nu}_{bac})$$

is the efficiency of the monochromator and detector system at the bacterial Raman scattering frequency

$$E(\bar{\nu}_{laser} - \bar{\nu}_{std})$$

is the efficiency of the monochromator and detector system at the standard Raman scattering frequency

C_{std} and C_{bac} are the molar concentration of the standard and bacteria

L(std) is the local field correction at the standard scattering frequency of the standard solution

$$= \left(\frac{n_{std}}{n'_{std}}\right)^2 \frac{\left(n^2_{std}+2\right)^2 \left(\left(n'_{std}\right)^2+2\right)^2}{81}$$

L(bac + std) is the local field correction at the standard scattering frequency of the solution of bacteria + standard

$$= \left(\frac{n_{bac+std}}{n'_{bac+std}}\right)^2 \frac{\left(n^2_{bac+std}+2\right)^2 \left(\left(n'_{bac+std}\right)^2+2\right)^2}{81}$$

$$\left(\frac{n_{std}}{n_{bac+std}}\right)^2$$

	is the correction for the solid angle of collection of Raman collection optics
(Self-Abs)	is the self-absorption correction factor, which depends upon the concentration and electronic absorption spectrum of the bacterial + standard solution
$\bar{\nu}_{laser}$	is the frequency of the excitation beam, in wavenumbers
$\bar{\nu}_{bac}$	is the frequency of the bacterial Raman scattering band, in wavenumbers
$\bar{\nu}_{std}$	is the frequency of the standard scattering band, in wavenumbers
n_{std}	is the index of refraction of the standard solution at $(\bar{\nu}_{laser} - \bar{\nu}_{std})$
n'_{std}	is the index of refraction of the standard solution at $\bar{\nu}_{laser}$
$n_{bac+std}$	is the index of refraction of the bacterial + standard solution at $(\bar{\nu}_{laser} - \bar{\nu}_{std})$
$n'_{bac+std}$	is the index of refraction of the bacterial + standard solution at $\bar{\nu}_{laser}$

LOCAL FIELD AND SOLID ANGLE OF COLLECTION CORRECTIONS

Equation 2 expresses in mathematical formalism, a way of calculating the absolute differential Raman cross-section (ADRCS) depicted in Figure 1. Equation 2 gives the solution independent cross-section of the analyte Raman bands, the cross-section of an isolated bacterium, out of solution, in the gas phase. This is accomplished by the inclusion of two factors in equation 2, the local field correction, and the solid angle correction. The local field correction 32 effectively removes the dependence of the strength of the electronic transition on solvent dielectric constant that affects the intensity of the Raman vibrational transitions. The solid angle correction 35, removes the solvent dependency of the solid angle of Raman light 12 collected with a given collection geometry. As the index of refraction of the bacteria-Raman intensity standard solution increases, the solid angle of Raman light 12 collected decreases. The solid angle correction factor compensates for this effect. Both of these factors require knowledge of the indices of refraction at the internal standard scattering frequency for the bacteria+Raman intensity standard solution and the Raman intensity standard solution, however these values are not known. If the Raman cross-sections of the bacteria are reported as values for the bacteria in the buffer used, and the index of refraction of the bacteria-Raman intensity standard solution does not differ significantly from the standard solution alone, the local field and solid angle correction 32, 35 can safely be neglected.

SELF-ABSORPTION CORRECTION

The self-absorption correction 34 depicted in Figure 1 must be considered to calculate the absolute differential Raman cross-section (ADRCS) according to equation 2. This correction factor 34 takes accounts of the absorption of the Raman light 12 by the sample. This factor is important when there are significant differences between the electronic absorption of the solution being irradiated at the laser excitation, the Raman intensity standard, and bacterial scattering frequencies. For the bacterial spectra, these frequencies usually span no more than 1700 cm^{-1}. When measuring the electronic absorption spectrum of a turbid sample such as a bacterial suspension, Rayleigh (elastically scattered) light must be rejected. When bacteria are mixed with beef menstrua that comprises blood and muscle cells, fecal material, and other biomatter, the acquisition of the electronic absorption spectrum is further complicated by these additional scatterers. For these reasons, special diffuse absorption apparatus or heads-on detectors may be used to minimize the effects of Rayleigh scattering so that an accurate absorption spectra can be obtained and applied to calculate the Raman cross-sections according to equation 2. However, with such a heterogeneous solution as beef menstrua and bacteria, the electronic absorption is not expected to vary significantly in any given 1700 cm^{-1} interval between 200 and 300 nm. In this event, the self-absorption correction factor can safely be neglected.

INSTRUMENT EFFICIENCY CORRECTION

The last correction factor 36 depicted in Figure 1 for the calculation of the absolute differential cross-section is the instrument efficiency correction 36. The efficiency of the monochromator 59 system was found not to vary more than 3% between 981 and 1700 cm^{-1} when exciting at 222 nm. Associated with the instrument efficiency correction 36, is the non-linearity of the response of the IPDA detector 72 across the length of the IPDA chip for a given photon flux and wavelength. This amounts to a detector efficiency correction. This non-linearity was corrected for by applying a gain curve correction. After the gain curve correction was applied for the detector 23, the efficiency of the monochromator-detector system in equation 2 is given by:

$$\frac{E(\bar{\nu}_{laser}-\bar{\nu}_{bac})}{E(\bar{\nu}_{laser}-\bar{\nu}_{std})} = 1$$

EQUATION USED TO CALCULATE THE ABSOLUTE RAMAN CROSS-SECTION

The equation used to calculate the absolute differential Raman cross-section depicted in Figure 1 is calculated in the following manner. Given that the efficiency factor is 1, and given the neglect of the local field 32, solid angle 35, and self-absorption 34 corrections, the cross-sections of BT and PF are calculated with the following revised equation:

Equation 3

$$\left[\frac{d\sigma}{d\Omega}\right]_{bac} = \left[\frac{d\sigma}{d\Omega}\right]_{std} \frac{I_{bac}}{I_{std}} \frac{C_{std}}{C_{bac}}$$

OPERATION

EXAMPLE I

BACTERIA IN H_20

The Raman system depicted in Figure 2 with intensified photodiode array (IPDA) detection was used to detect the sample Raman light 12, the gate width was set to 150 ns, the delay time was 500 ns, the repetition rate of the laser was typically 200 Hz. The spectra were produced by the addition of 25 60 second exposures of the detector. The concentrations for BT and PF were estimated from their optical densities measured at 600 nm to be in the range of 2-50 million bacteria/ml.

Figure 7 shows the 222 nm excitation spectra of (a) BT in water buffer, (b) PF in water buffer, and (c) water buffer. The only band that can be unambiguously assigned to bacterial scattering is the 1048 cm^{-1} band for both BT and PF. The BT band is much broader than the PF band centered at 1048 cm^{-1}.

EXAMPLE II

DEPOLARIZATION RATIO OF THE 1048 cm^{-1} BAND OF BT

The Raman system depicted in Figure 2 with intensified photodiode array (IPDA) detection was used to detect the Raman light 12, the gate width was set to 150 ns, the delay time was 500 ns, the repetition rate of the laser was typically 200 Hz. The spectra were produced by the addition of 25 60 second exposures of the detector.

Figure 8 shows the (a) measured total intensity, (b) parallel, and (c) corrected perpendicular component spectra of BT at 222 nm excitation. Since there is no detectable intensity in the perpendicular component in the 1048 cm^{-1} band, its depolarization ratio is very close to 0.

EXAMPLE III

EFFECTS OF HYDROGEN-DEUTERIUM EXCHANGE ON THE RESONANCE RAMAN SPECTRA OF BT AND PF

The Raman system 1 depicted in Figure 1 with intensified photodiode array (IPDA) detection was used to detect the sample Raman light 12, the gate width was set to 150 ns, the delay time was 500 ns, the repetition rate of the laser was typically 200 Hz. The spectra were produced by the addition of 25 60 second exposures of the detector.

Figure 9 shows the sensitivity of the resonance Raman spectrum of BT to the effects of hydrogen-deuterium (H-D) exchange. Changes in the resonance Raman spectra of BT are observed when 2.0 ml of stock BT solution are mixed with 10.0 ml of 0.15 M Na_2SO_4, 0.20 M Na_2HPO_4, pD 7.25 in heavy water (D_2O). The BT stock was quickly mixed with the buffer and the resonance Raman spectrum (a) was collected with a 25 minute integration time. The BT band at 1048 cm^{-1} observed in H_2O buffer, is not present in this spectrum. The solution was allowed to sit at room temperature for 9.5 hours, after which the spectrum (b) was collected. A band was found in the new position of 1044 cm^{-1} which was neither present in the spectrum collected after (a) 25 minutes of exchange, nor in that of the (c) H_2O solution.

Figure 10 shows the sensitivity of the resonance Raman spectrum of PF to the effects of hydrogen-deuterium (H-D) exchange. Changes in the resonance Raman spectra of PF are observed when 2.0 ml of stock PF solution are mixed with 10.0 ml of 0.15 M Na_2SO_4, 0.20 M Na_2HPO_4, pD 7.25 in heavy water (D_2O). The PF stock was quickly mixed with the buffer and the resonance Raman spectrum (a) was collected with a 25 minute integration time. The PF band at 1048 cm^{-1} observed in H_2O buffer, is not present in this spectrum. The solution was allowed to sit at room temperature for 9.5 hours, after which the spectrum (b) was recollected. A band in the new position of 1050 cm^{-1} not present in the spectrum collected after (a) 25 minutes of exchange nor present in the (c) H_2O solution was observed.

Figure 11 shows the resonance Raman spectra of (a) BT and (b) PF that have been subjected to 9.5 hours of (H-D) exchange. After exchange, the 1048 cm^{-1} band, the band initially observed, disappears but a new band reappears for both BT and PF. The new band reappears at the lower frequency of 1044 cm^{-1} for BT and at the higher frequency of 1050 cm^{-1} for PF.

The data in Figures 9, 10 and 11 show that the 222 nm excitation resonance Raman spectra of BT and PF can be used to distinguish between these two species if these bacteria are subjected to H-D exchange. The H-D exchange process causes the 1044 cm^{-1} bands that both species exhibit to shift their wavenumber position in different directions, as well as modulating the intensities of these bands as a function of time. The absolute differential Raman cross-sections of BT and PF are given in the table below. These observations form the basis for the identification and quantification of bacteria.

TABLE 1

Absolute Differential Raman Cross-Sections of Bacteria Brocothrix thermosphacta and Pseudomonas fluorescens, 222 nm laser excitation				
Height of the SO_4^{-2} 981 cm^{-1} band = 1.00				
2-50 x 10^6 bacteria/ml				
Bacterium	**H-D Exchange time (Hours)**	**Frequency cm^{-1}**	**Height**	**Cross Section, cm^2/ (bacteria-steradian)**
BT (H_2O)	-	1048	0.027	1.2-30 x 10^{-17}
BT (D_2O)	.3	1044	0	0
BT (D_2O)	9.8	1044	0.014	0.60-15x10^{-17}
PF (H_2O)	-	1048	0.030	1.3-33x10^{-17}
PF (D_2O)	.3	1050	0	0
PF (D_2O)	9.8	1050	0.013	0.56-14x10^{-17}

EXAMPLE IV

EFFECTS OF HYDROGEN-DEUTERIUM EXCHANGE ON THE RESONANCE RAMAN SPECTRA OF THE BEEF MENSTRUA-BACTERIA SOLUTION

The Raman system using the intensified charge couple device (ICCD) detection was used to detect the Raman light 12. The repetition rate of the laser was typically 200 Hz. The spectra were produced by the addition of 25-50 20 second exposures of the detector. Figure 12 shows the effects of H-D exchange on the resonance Raman spectra of

beef menstrua to which bacteria were added. This sample was prepared by adding 3.3 ml of beef menstrua to 6.7 ml of either the 0.15 M Na_2SO_4, 0.20 M Na_2HPO_4 , pD 7.25 in heavy water (D_2O) or 0.15 M Na_2SO_4, 0.20 M Na_2HPO_4 pH 7.25 in water (H_2O) . The total bacteria concentration is 24,000 bacteria/ml estimated from an ATPase activity test. The bacteria were a mixture of bacteria that are found on beef carcasses. The beef menstrua-bacteria solution was observed to contain bone and tissue fragments and was opaque with red cow blood. The rectangular box on the left encompasses the 981 cm^{-1} mode of the internal standard, SO_4^{-2}. The uppermost spectrum in Figure 12 is that of beef menstrua-bacteria in the H_2O based buffer. This spectrum serves as the reference spectrum to judge the effects of H-D exchange on the sample. The spectra below this spectrum show the time dependent effects of H-D exchange on the resonance Raman spectra acquired 15, 21, 29, 89, 100, 107, and 117 minutes after mixing the beef menstrua with the D_2O buffer. Proceeding from low to high frequency, the following changes are noted in these spectra. The dashed line at 1003 cm^{-1} indicates the position of a maximum of a band from the beef menstrua-bacteria solution in H_2O buffer. This maximum shifts to 1007 cm^{-1} after 117 minutes of H-D exchange. There is a continuous decrease in the height of this band relative to the height of the sulfate standard at 981 cm^{-1} as the H-D exchange process proceeds. A new band at 1054 cm^{-1}, not present in the H_2O based buffer, acquires significant intensity in the D_2O buffer. Similarly, a very weak band in the H_2O buffer at 1151 cm^{-1}, also grows significantly in intensity as a result of the H-D exchange process. New bands present in the 117 minute D_2O spectrum not present in the H_2O spectrum are found at 1247, 1387, 1441, 1544, 1572 cm^{-1}. These bands can also be found in spectra acquired in the D_2O buffer earlier. Since all spectra are normalized to the height of the sulfate standard at 981 cm^{-1}, the height of each band is equivalent to the ratio of the height of each band to the height of the sulfate band that serves as the internal intensity standard. The spectra presented in this format allow one to follow both relative (between successive Raman spectra) and absolute (relative to the sulfate height or area) changes in the Raman spectra. These observations forms the basis for the third step in Figure 1, the identification and quantification of bacteria.

Figure 13 summarizes some of the changes in the time dependent behavior of the resonance Raman spectra of the beef menstrua-bacteria in D_2O buffer. The heights of the 1003-7 and 1147 cm^{-1} bands, normalized to their values in H_2O buffer, are plotted as a function of time. Also shown in this graph is the change height of the 1051 cm^{-1} as a function of time. This figure unambiguously shows that these H-D exchange induced changes in the heights of the Raman bands, relative to the absolute Raman cross-section standard, occur at different rates. These observations form the basis for the identification and quantification of bacteria.

Figure 14 shows the spectra of the beef menstrua-bacteria, PF, and BT samples in D_2O buffer. The dashed line indicates the position of the vibrational band associated with the BT bacteria at 1048 cm^{-1}, as well as its position relative to the PF band at 1043 cm^{-1}, and a beef menstrua-bacteria band at 1054 cm^{-1}. These three bands are present at these positions only when the samples are subjected to H-D exchange.

RAMIFICATIONS AND SCOPE

The invention provides a novel and nonobvious bacterial identification and quantification system using resonance Raman spectroscopy that provides quantitative information, that is instrument independent, requires no special sample preparation, reduces spectral artifacts, has the potential of discriminating between pathogenic and non-pathogenic bacteria, provides data faster than classical bacteriological test, and is economical per test.

The determination of the differential Raman cross-sections of bacteria as depicted in Figure 1, and extracted from the data in Figures 7, 9, and 10 using equation 3, and summarized in Table 1, are described for the first time herein. This establishes a resonance Raman spectral titer for bacteria in the buffers used. From the cross-section values reported above, and the relative heights of sulfate to bacterial Raman bands, one can use equation 3 to calculate the number of bacteria/ml in the sample.

From the cross-section values reported above, the sulfate absolute differential Raman cross-section standard, and the relative heights of sulfate to bacterial Raman bands, one uses equation 3 to solve for C_{bac}, the number of bacteria/ml in the sample.

Using equation 3, the absolute differential Raman cross-sections of these Raman bands is calculated once the concentration of the species responsible for the appearance of these vibrational bands is determined.

The relationship depicted in Figure 1 between absolute differential Raman cross-sections of bacteria and environmental perturbations is understood by considering the data in Figures 9 and 10 and Table 1. The following conclusion can be drawn for the Raman bands observed for BT and PF in the buffers used: their absolute differential Raman cross-sections are different in D_2O than H_2O buffer, that their absolute differential Raman cross-sections in D_2O change as a function of H-D exchange time, and that the BT and PF Raman bands shift their frequency in different directions upon H-D exchange. Because all bacteria are comprised of proteins, fats, and nucleic acids, it is reasonable to expect all bacteria to exhibit time dependent changes in the intensity and frequency of some of their vibrational bands in their resonance Raman spectra when undergoing H-D exchange, and excited at the appropriate excitation frequency. These observations and conclusions form the basis of the claim that environmental perturbations, in the form of H-D exchange

induced changes in the absolute differential Raman cross-sections of the vibrational bands of bacteria, facilitate the identification and quantification of bacteria.

The invention combines the spectral selectivity of resonance Raman spectroscopy and the physical-chemical selectivity of H-D exchange. These two selection mechanisms allow one to preferentially observe and change the vibrational bands associated with proteins and nucleic acid constituents. H-D exchange affords selectivity, via manipulation of external physicochemical parameters, to control the nucleic acid : nucleotide to protein relative exchange rates, and thereby the relative extent of nucleic acid : nucleotide and protein deuteration. The deuteration and the extent of deuteration can be observed with Raman spectroscopy. By choosing the appropriate excitation frequency, either nucleic acids or proteins are preferentially enhanced.

This manipulation of H-D exchange rate and excitation frequency is useful in establishing the identity of the constituents in a heterogeneous sample containing different components, e.g. different types of bacteria. If the spectra of two bacterial species are very similar at a given excitation frequency one can subject the sample containing the two bacterial species to H-D exchange under conditions such that their spectra change in different, predetermined ways. This facilitates the identification and quantitation of the bacteria and analytes in question, in a manner that would not be possible with the static, non-H-D exchange picture.

The relationship between the absolute differential Raman cross-sections of beef menstrua-bacteria and environmental perturbations may be understood by considering the data in Figures 13 and 14. Figure 13 shows that the heights of the Raman bands of the beef menstrua-bacteria solution (relative to an absolute Raman cross-section standard) change at different rates as a function of H-D exchange time. Using equation 3, the absolute differential Raman cross-sections of these Raman bands can be calculated once the concentration of the species responsible for the appearance of these vibrational bands is determined. The following conclusion can be drawn for the beef menstrua-bacteria sample: the absolute differential Raman cross-sections of some of its vibrational bands are different in D_2O than H_2O buffer; some of these absolute differential Raman cross-sections in D_2O change as a function of H-D exchange time; and some of these Raman bands shift their frequency upon H-D exchange. The H-D exchange induced changes in the absolute differential Raman cross-sections observed in the resonance Raman spectra of the mixture of bacterial species and beef menstrua exhibit the expected time dependent behavior of biomolecules with exchangeable protons that have different accessibility to solvent. Some of the Raman bands in the spectrum of the bacteria-menstrua mixture probably belong to bacteria, and some to extra-bacterial biomolecular constituents of the beef menstrua. The time dependent changes in the Raman spectra of a bacteria-free beef menstrua solution, with respect to an internal standard, would be useful in identification of the characteristic Raman band profiles or "spectral signatures" of the extra-bacterial components. These observations and conclusions form the basis of the claim that environmental perturbations, in the form of H-D exchange induced changes in the absolute differential Raman cross-sections of the vibrational bands of a beef menstrua-bacteria solution, facilitate the identification and quantification of the bacteria in beef menstrua.

Figure 14 compares the (a) BT, (b) PF, and (c) beef menstrua-bacteria resonance Raman spectra. It is observed that all samples exhibit a band in the 1049 ± 5 cm^{-1} region that is sensitive to the H-D exchange time. Whether or not this band observed in the beef menstrua-bacteria solution is indeed a bacterial vibrational band, the Raman spectra of each of the bacteria species has to be individually collected, and their spectra compared to the spectra obtained from the beef menstrua-bacteria solution.

The depolarization ratio of Raman bands may be a taxonomic of a bacterium and extra-bacterial components. A depolarization ratio of 0 has been measured for BT at 1048 cm^{-1} in the buffer at 222 nm excitation. It has been shown that this observable can vary dramatically with excitation frequency for the peptide bond, this observable is sensitive to the environment and number of excited and/or ground states contributing to the resonance Raman scattering process. See Figure 15.

GAIN CURVE CORRECTION

A sandblasted quartz plate was placed immediately in front of the detector to act as a light diffuser so as to evenly expose the detector. A deuterium lamp, 0.5 m from the detector, generated UV light that was filtered through a band pass filter 300 ± 15 nm exposing the detector. Detector parameters we set to be identical to those used during the collection of Raman data, with the exception of total exposure time. The total exposure time was set so as to bring the collected counts for a single scan to be approximately 14,000. One thousand scans of the UV lamp were signal averaged to produce a measured gain curve spectrum while one thousand dark current scans were signal average to produce dark current spectrum. The dark current spectrum was subtracted from the measured gain curve spectrum, with both regions on the edge of the detector not intensified truncated away to produce a calculated gain curve spectrum. The calculated gain curve spectrum was divided by the count reading at pixel 512, to produce a final gain curve correction spectrum.

The observed experimental Raman spectra were divided by the final gain curve spectrum to produce gain curve corrected Raman spectra.

SYSTEM CONSIDERATIONS

SAMPLE HANDLING AND PREPARATION

The introduction of the of the new sampling handling apparatus depicted in Figure 4 reduces the likelihood that the biomolecules in the sample will denature due to interactions with extensive quartz surfaces found in the quartz capillary tubes previously used, and reduces the likelihood of photodecomposition of the sample and the quartz in the area that the laser impinges upon. This reduction of the risk of photodecomposition of the sample and elimination of the photodecomposition of the sample container reduces the likelihood of laser induced spectral artifacts. It has been shown with pure cultures of bacteria and mixtures of bacteria with beef menstrua that no special sample preparation is required to acquire resonance Raman spectra.

This sample handling technique may be used even without the deuteration step and thus, viewed from an alternative aspect, the invention provides a method of identifying an analyte, preferably a biological contaminant such as bacteria, viruses, yeasts, fungi or prions, in a liquid sample said method comprising:

i) directing monochromatic light, e.g. a laser beam, at an exposed moving surface of said sample;
ii) detecting Raman light signal from said sample;
iii) and comparing said Raman light signal with a standard whereby to identify said analyte.

In this method, the moving surface is moving relative to optical axis of the monochromatic light impinging thereon and is preferably a substantially planar vertical surface, e.g. a falling curtain of the fluid sample.

DEPOLARIZATION AND ABSOLUTE RAMAN CROSS-SECTION STANDARDS

Figure 1 depicts a bacterial quantification and identification system with which a single absolute Raman intensity standard has been used, the SO_4^{-2} ion. These experiments could be carried out with any absolute differential Raman cross-section standard compatible with the requirements of the bacteria, pH, solubility, ionic strength, etc. Absolute intensity standards have been measured for a number of inorganic and organic species. The use of these species for depolarization ratio standards means that that this value should be known for at least one of the vibrational bands of the Raman standard at the laser excitation frequency used.

The depolarization ratio is sensitive to changes in the excitation frequency, environmental conditions, number of excited states; because of this multiple sensitivity, it is reasonable to expect that the depolarization ratio will facilitate the identification (and possibly the quantification) of bacteria. The depolarization ratio is probably more sensitive to changes in the excitation frequency, environmental conditions, number of excited states than the absolute differential Raman cross-section.

The change in magnitude of this observable as a function of excitation frequency, buffer, H-D exchange time, and other environmental parameters may also facilitate the unique taxonomic identification of bacteria and extra-bacterial components.

ESTIMATED TIME TO ACQUIRE A RESONANCE RAMAN SPECTRUM

The time it takes to acquire Raman data in the method of the invention depends upon the efficiency of the monochromator and detectors used, and the laser power incident on the sample. Given the efficiency of a state of the art system optimized to acquire resonance Raman spectra, and the use of a continuous wave laser excitation source, at least a 100 fold increase in the data collection speed can be obtained. Although the description above contains many specificities, these should not be construed as limiting the scope of the invention but as merely providing illustrations of some of the presently preferred embodiments of this invention. Various other embodiments and ramifications are possible. For example, the following the following ramifications are considered.

NOVEL QUANTITATIVE INFORMATION REQUIRES COMPUTER PROCESSING ALGORITHMS FOR EFFICIENT BACTERIAL ANALYSIS

To efficiently identify and quantify bacteria in samples, the resonance Raman data collected will conveniently be analyzed with computerized algorithms utilizing the spectra of known bacteria.

INDUSTRIAL APPLICATION REQUIRES INSTRUMENT INDEPENDENT DATA

Figure 17 shows two situations where Raman data is collected from different instruments, (a) using depolarizer

19 in the Raman collection optics 35 and (b) without the depolarizer 19 in the Raman collection optics 54. In situation (a) a single spectral library or artificial neural network can be used to analyze the data from the non-identical Raman spectrometers, whereas in (b), because depolarizer 19 is absent, the data from each of the non-identical Raman spectrometers must be processed with dedicated spectral libraries or artificial neural networks that are specific for each of the Raman spectrometers. With the inclusion of the correction factors depicted in Figure 1, and the depolarizer 19 in front of the monochromator 59 in Figures 2 and 3, the information acquired by the method of the invention is instrument independent, that is, detector, grating, and monochromator bias have been removed. The importance of this feature will become evident upon consideration of the following methods of computerized spectral recognition.

SEQUENTIAL LOOKUP IN DATABASE

These algorithms in their simplest form entail comparing the resonance Raman spectrum or spectra of the sample containing the bacteria to be identified to known spectra of bacteria stored in a spectral database. It is envisioned that the data in this database will be collected by a single Raman spectrometer and that the spectra will be collected at many excitation frequencies for a variety of bacteria. This computerized database will be employed by a plurality of distinct Raman spectrometers for bacterial identification and quantification, each with non-identical detector, grating, and monochromator biases. The successful identification and quantification of bacteria using this single computer database by a plurality of distinct Raman spectrometers necessitates that the data collected by each Raman spectrometer be instrument independent. If this were not so, a dedicated spectral database would have to be generated for each Raman spectromator with a specific detector, grating, and monochromator combination. This would make the identification and quantification of bacteria in an industrial environment significantly less practical. Thus, a single database may be utilized by all Raman spectrometers to identify and quantify bacteria provided that the procedures and designs described herein are followed.

ARTIFICIAL NEURAL NETWORKS

Artificial neural network algorithms offer alternatives to the method of identification and quantification of bacteria discussed above, the comparison of the spectrum or spectra of a bacterial sample to the known spectra of bacteria in a database. This method involves time consuming look-ups through the database. Neural networks can be trained to associate a substance to its spectrum through presentation of the known spectra to the neural network using special learning paradigms. Once the artificial neural network has been trained, the spectral recognition process of an unknown spectrum can be much faster than the time consuming look-up procedure mentioned above. Besides the increased recognition speed of the artificial neural network algorithms, these algorithms can function with noisy spectra. Often a prerequisite for optimal artificial neural network processing, the normalization of the spectra used to train the artificial neural network is facilitated by the Raman bands associated with the internal absolute differential Raman cross section scattering standard.

A NOVEL SYSTEM FOR THE TAXONOMIC IDENTIFICATION OF BACTERIA

Figure 1 depicts a novel system for the identification and quantification of bacteria. It is shown here that the absolute differential Raman cross-sections of bacterial Raman bands are sensitive to the H-D exchange process; although the experiments reported herein have been limited to a single pH/D condition, the invention is not limited to this single pH. The H-D exchange process is sensitive to pH, where solvent accessible protons exchange at a faster rate than other protons, that biomolecules such as nucleic acids and proteins exchange at different rates at a given pH/D, and that these differences in exchange rate can occur over many orders of magnitude with respect to pH/D.

It has also been shown that the addition of biomolecular denaturants, such as high salt concentrations can change the structure of proteins and nucleic acids, thus altering which chromophores are accessible to solvent, changing their H-D exchange rate, and that may in turn affect the time dependent resonance Raman spectra of the bacteria. Similarly, heating or cooling the bacteria to change the structure of the biomolecular constituents that in turn change the population of accessible exchangeable protons, is also expected to affect the resonance Raman spectra of these species undergoing H-D exchange. Thus, this invention covers all forms of environmental perturbations of the biomolecular constituents of the bacteria to produce changes in the resonance Raman spectra of these species undergoing H-D exchange. Factors - denaturant, temperature, excitation frequencies, exchange rate - can be varied to improve quantitation and/or identification.

RESONANCE RAMAN RESPONSE SPECTRUM

Figure 16 depicts the resonance Raman response profile. It is reasonable to expect that via manipulation of the

one or more environmental parameters mentioned above, monitored as a function of time during the H-D exchange process, characteristic time dependent Raman spectra, a resonance Raman response profile, can be collected and stored in a computer that uniquely or nearly uniquely describes the species or sub-species of any bacterium. When analyzing a complicated mixture of bacteria and beef menstrua for the presence of a specific bacterial species or sub-species, the mixture can be subjected to the conditions used to produce the response profile for the species or sub-species in question, and compared to the stored response profile, to determine whether or not the bacterial species is present or not. What distinguishes this method from other spectroscopic identification and quantification methods is the manipulation of the environment to induce changes in the resonance Raman spectrum of an unknown sample, and to compare these changes to the reproducible predetermined changes in the Raman spectra of bacteria or biomolecule already studied. Thus, identification and quantification of bacteria and biomolecules is not a static process, but rather, a dynamic one. Factors denaturant, temperature, excitation frequencies, exchange rate - can be varied to improve quantitation and/or identification.

MULTIPLE EXCITATION FREQUENCIES AND RESONANCE RAMAN RESPONSE SPECTRA

If the Raman response profile yields ambiguous results at the excitation frequency used, another excitation frequency can be used. It has been shown that resonance Raman spectra collected at different excitation frequencies exhibit preferential enhancement of protein or nucleic acid constituents; thus at 222 nm excitation bacterial protein vibrational bands are enhanced, while at 240 nm excitation, bacterial nucleic acid are enhanced. The combination of excitation frequency and the time dependent effects of the H-D exchange process (with or without environmental perturbation) on the resonance Raman spectrum, may allow an effectively unique resonance Raman taxonomic description of any bacterium to be obtained.

GENERAL BIOMOLECULAR IDENTIFICATION - VIRUSES, TISSUES, ETC

Although this invention is particularly aimed at addressing the identification of meat and poultry bacterial pathogens, it is not be limited to bacterial pathogens found on these substrates. This invention is useful in the identification and quantification of any biomolecular assembly found on any substrate, including viruses and fungi. The substrate could include and biomolecular tissue or any substrate including walls, tabletops, cooking utensils, etc.

USE IN NONBIOLOGICAL SYSTEMS THAT HAVE EXCHANGEABLE PROTONS

The time dependence of the exchange of proton for deuterons manifested in the resonance Raman spectrum or spectra of the species in question, coupled with the excitation frequency dependence of the resonance Raman spectrum may used to quantify and identify any species with exchangeable protons, such as plastics and inorganic materials.

It will be appreciate by those of ordinary skill in the art that various changes and modifications may be made to the description and drawings without departing from the spirit and scope of the present invention. For example, whereas the Raman collection geometry discussed above is a backscattering, other geometries could be employed to acquire the Raman spectra 22.

Claims

1. A method of identifying an analyte in a sample, said method comprising:

   i) treating said sample with a deuterating agent;
   ii) directing monochromatic light at said sample;
   iii) detecting Raman light signal from said sample;
   iv) and comparing said Raman light signal with a standard whereby to identify said analyte.

2. A method as claimed in claim 1 wherein said deuterating agent is D_2O.

3. A method as claimed in claim 1 or claim 2 wherein said analyte is a biological contaminant.

4. A method as claimed in any one of claims 1 to 3 wherein said analyte is selected from bacteria, viruses, yeasts and fungi, fragments thereof and products thereof.

5. A method as claimed in any one of claims 1 to 4 wherein said sample comprises body fluid, cells, tissues, or waste

from a human or non-human animal body.

6. A method as claimed in any one of claims 1 to 5 wherein said Raman light signal is subject to depolarization and wavelength separation before detection.

7. A method as claimed in any one of claims 1 to 6 wherein said sample contains a reference material exhibiting a predetermined characteristic Raman light signal.

8. A method as claimed in any one of claims 1 to 7 wherein a plurality of Raman light signals are detected for said sample at different extents of deuteration of analytes therein.

9. A method as claimed in claim 8 wherein the different extents of deuteration are produced by deuteration at different temperatures.

10. A method as claimed in claim 8 wherein the different extents of deuteration are produced by deuteration at different pHs.

11. A method as claimed in any one of claims 1 to 10 wherein a plurality of Raman light signals are detected for said sample using incident monochromatic light of different wavelengths.

12. A method as claimed in any one of claims 1 to 11 wherein said sample is contacted with a denaturant.

13. A method as claimed in any one of claims 1 to 12 wherein said monochromatic light is directed at a substantially planar exposed moving surface of a liquid sample.

14. A method as claimed in any one of claims 1 to 13 wherein the detector used to detect said Raman light signal is so disposed as to substantially avoid detection of Rayleigh scattering of said monochromatic light.

15. A method as claimed in any one of claims 1 to 14 wherein comparison of said Raman light signal with a standard involves comparing with predetermined characteristic Raman spectral data for one or more potential analytes also subjected to treatment with the deuterating agent, whereby to determine the presence in or absence from said sample of one or more said potential analytes and/or to determine the concentration in said sample of one or more said potential analytes.

16. A method as claimed in any one of claims 1 to 15 of resonance Raman spectroscopy for identifying and/or quantitating proton-bearing analytes including bacteria, viruses, prions, biomolecules, biomolecular assemblies, inorganic and organic compounds comprising:

deuterating with dideuterium oxide (D_2O) a sample containing one or more analytes, each analyte having exchangeable protons such that said deuteration will cause the protons of said analyte to be exchanged with deuterons;
providing a monochromatic light at an excitation frequency onto a sample for producing Raman sample light and rejecting Rayleigh light;
passing said Raman sample light through a depolarizer for producing randomized polarization components of said Raman sample light;
generating a Raman sample spectrum calibrated with respect to an absolute differential Raman cross-section standard in response to said randomized polarization components;
providing said Raman sample spectrum to a spectral analyzer;
providing the identity and/or quantity of analytes in the sample.

17. A method as claimed in claim 16 wherein the step of providing the identity and/or quantity of the analytes in the sample includes one or more of the steps of:

performing an instrument efficiency correction in response to the Raman sample spectrum;
performing a self absorption correction in response to the Raman sample spectrum;
performing a solid angle correction in response to the Raman sample spectrum; and
performing a local-field correction in response to the Raman sample spectrum.

18. A method as claimed in either of claims 16 and 17, further comprising passing said Raman light through a polarizer for getting a perpendicular component spectrum and a parallel component spectrum for providing a depolarization ratio of a Raman analyte band for the identification of said analytes, said perpendicular components optionally being provided in response to a calculated, corrected perpendicular component for providing a depolarization ratio of a Raman analyte band for the identification of said analytes.

19. A method as claimed in any one of claims 1 to 18 wherein said sample is retained in a sampling apparatus, comprising:

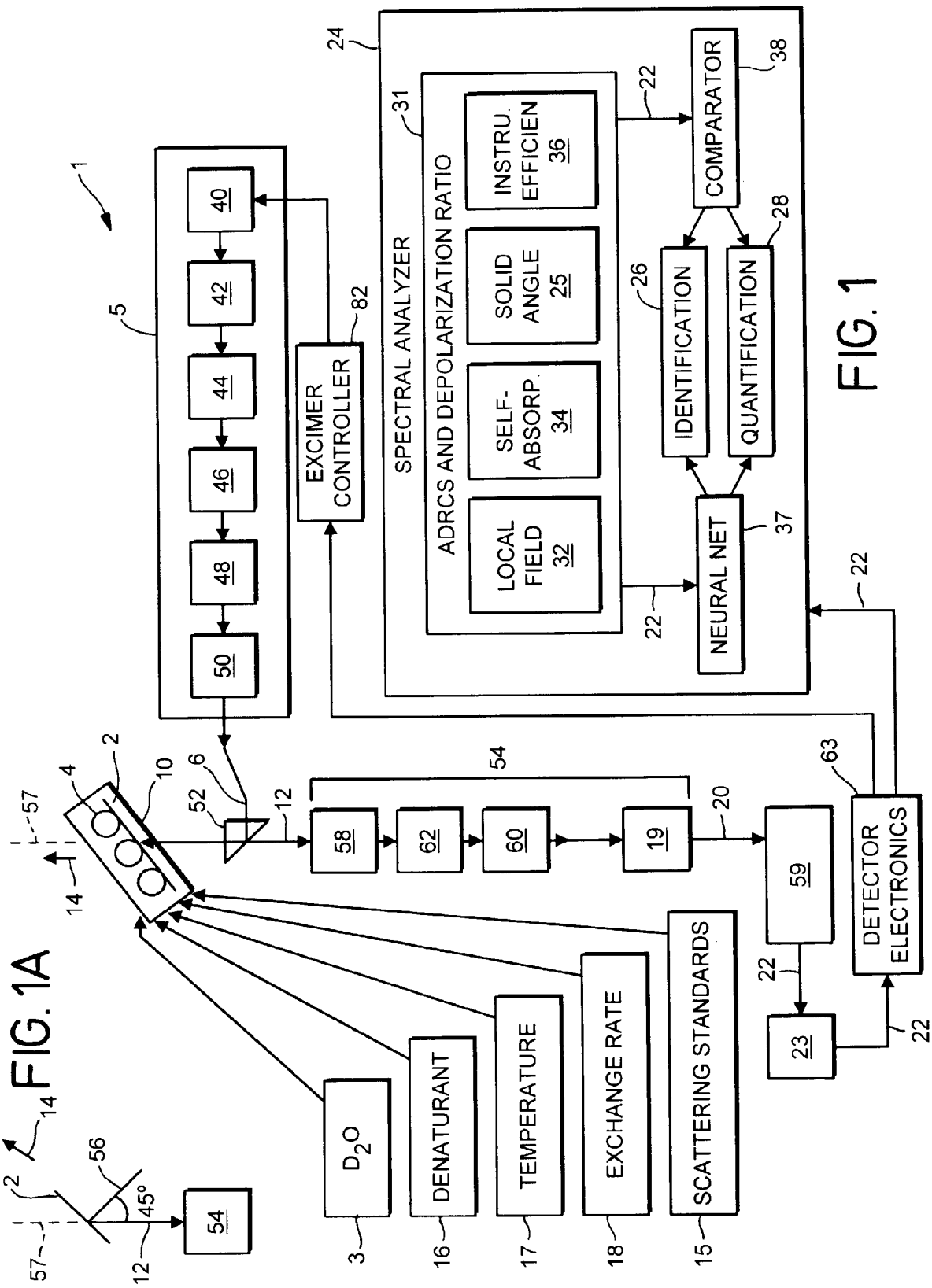
a reservoir for said sample;
a pump for continuously drawing said analyte; a planar flow device having a windowless sample stream between a pair of bookends said pair having a first end and a second end such that said analyte is continously flowing from said pump into the first end, between said bookends for being exposed to said monochromatic light, wherein the angle of the normal of said windowless sample stream is beteween 0 and 90 degrees with respect to the collection optic for rejecting Rayleigh scattering, and exiting said planar flow device.

20. An apparatus for use in a method as claimed in claim 1 said apparatus comprising:

i) a reservoir (100) for a sample (2);
ii) pump means (102) for creating a continuous flow of said sample in a light exposure zone (93) in which the flowing sample is not in contact with a container wall;
iii) means (5) for directing a beam of monochromatic light onto the surface of said sample in said light exposure zone;
iv) means (23) for detecting Raman light from said sample in said light exposure zone, said means for detecting being disposed so as substantially not to detect Rayleigh scattered light from said sample; and optionally
v) depolarization means (19) disposed to depolarize Raman light from said sample before wavelength separation and detection thereof.

21. A sample holder for a spectrometer comprising:

i) a reservoir (100) for a liquid sample (2);
ii) pump means (102) for pumping said sample from said reservoir to a planar flow device (95) having a downwardly-opening horizontally-elongate aperture with downwardly- extending planar-flow edge-defining means (97, 98) at the ends thereof; and optionally
iii) funnel means (99) for receiving liquid sample flowing from said aperture and returning said liquid sample to said reservoir.


1
5
40
42
44
46
48
50
82
EXCIMER CONTROLLER
24
SPECTRAL ANALYZER
31
ADRCS AND DEPOLARIZATION RATIO
LOCAL FIELD 32
SELF-ABSORP. 34
SOLID ANGLE 25
INSTRU. EFFICIEN 36
22
COMPARATOR
38
IDENTIFICATION
26
QUANTIFICATION
28
NEURAL NET
37
22
22
2
4
10
6
52
12
57
14
58
62
60
19
54
20
59
63
DETECTOR ELECTRONICS
22
23
22
D_2O
3
DENATURANT
16
TEMPERATURE
17
EXCHANGE RATE
18
SCATTERING STANDARDS
15
FIG. 1
FIG. 1A
14
2
57
56
45°
12
54

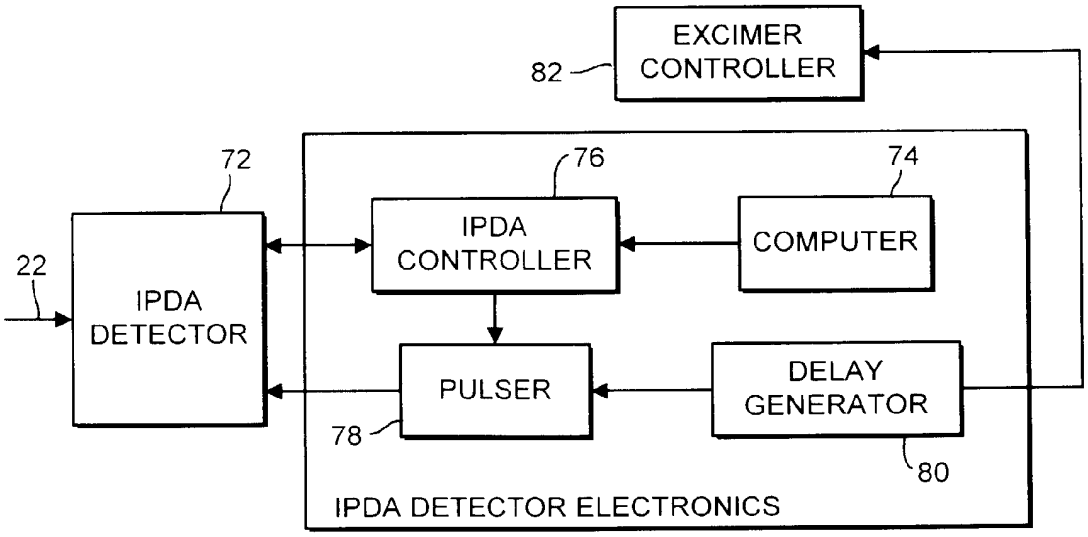

EXCIMER CONTROLLER
82
72
76
74
22
IPDA DETECTOR
IPDA CONTROLLER
COMPUTER
PULSER
DELAY GENERATOR
78
80
IPDA DETECTOR ELECTRONICS

FIG. 2

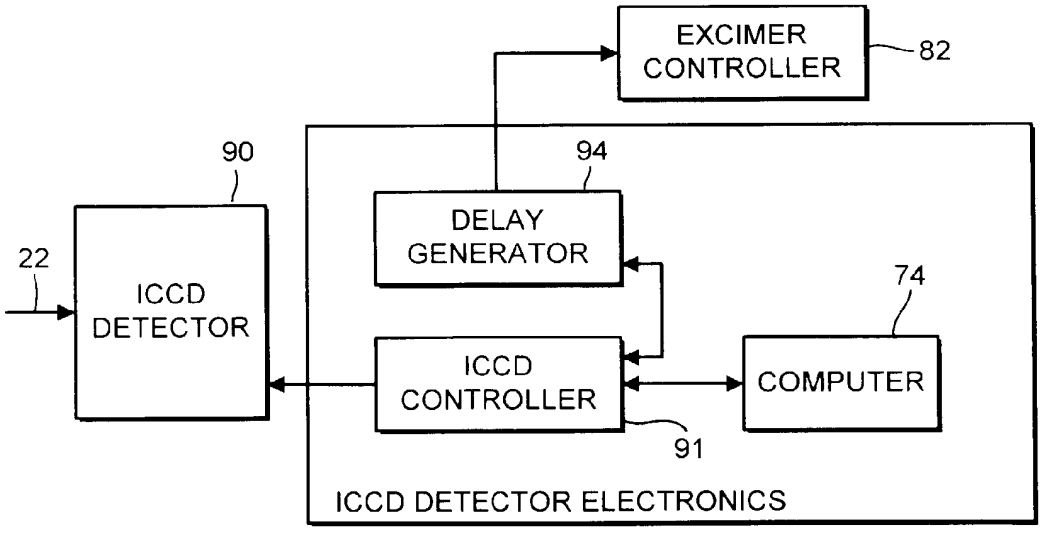

EXCIMER CONTROLLER
82
90
94
22
ICCD DETECTOR
DELAY GENERATOR
74
ICCD CONTROLLER
COMPUTER
91
ICCD DETECTOR ELECTRONICS

FIG. 3

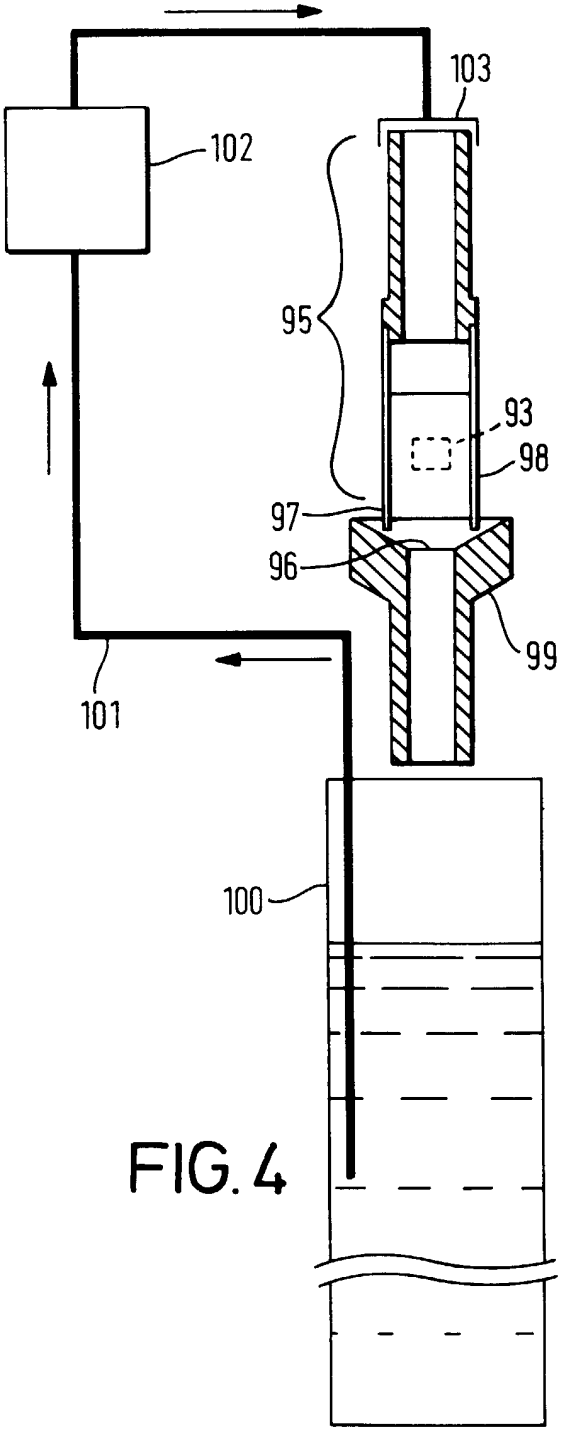

103
102
95
93
98
97
96
99
101
100

FIG. 4

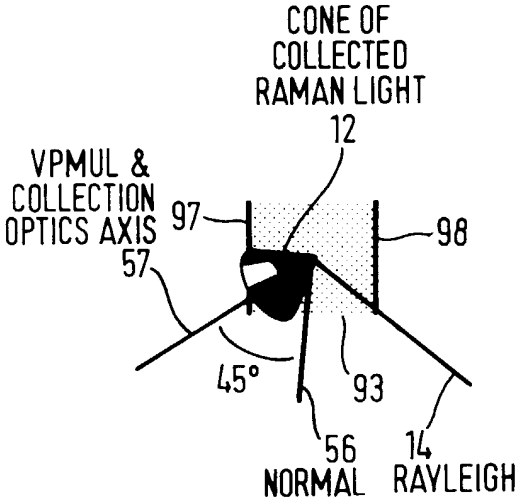

CONE OF
COLLECTED
RAMAN LIGHT
12
VPMUL &
COLLECTION
OPTICS AXIS
97
98
57
45°
93
56
NORMAL
14
RAYLEIGH

FIG. 4A

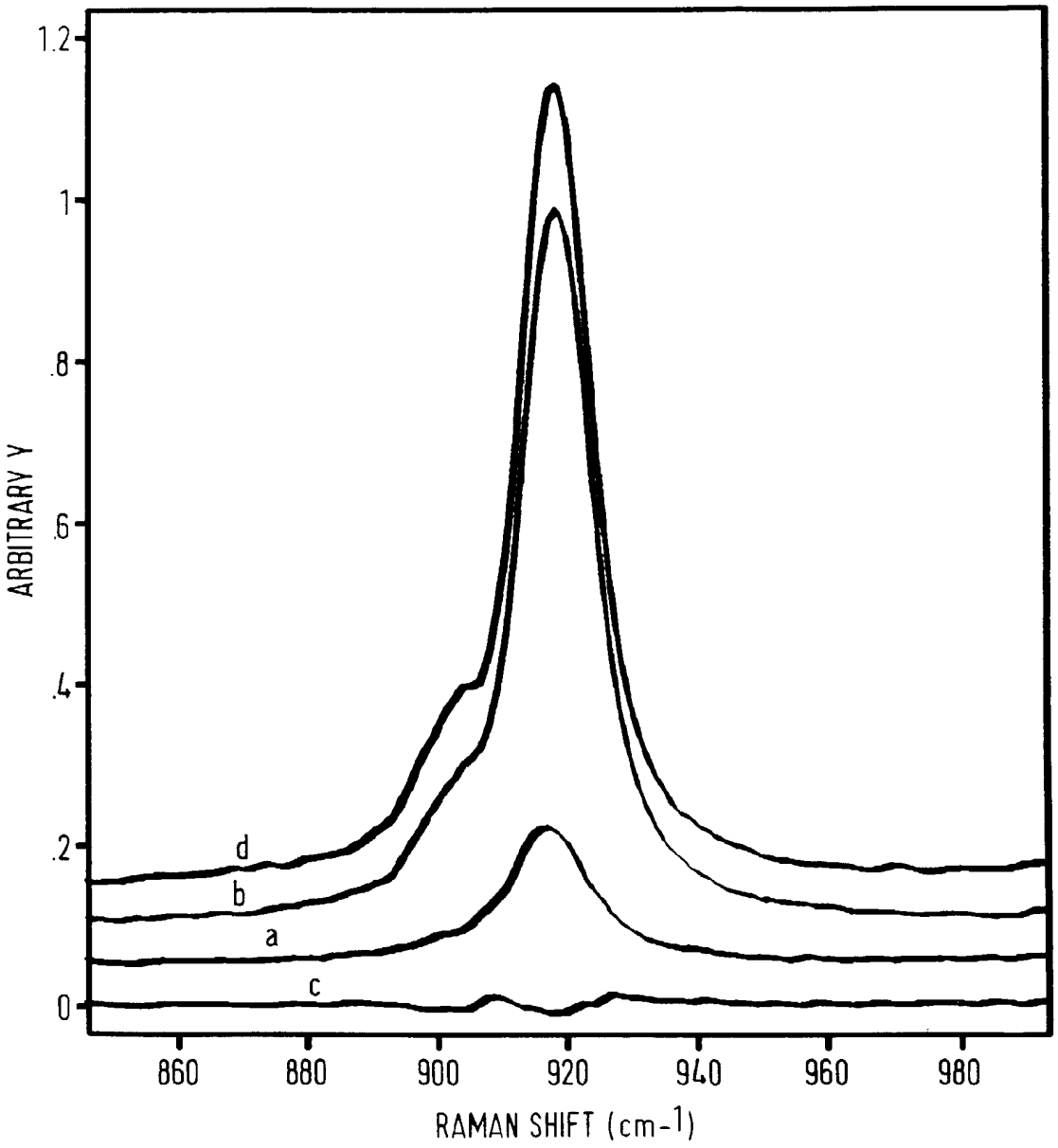

1.2
1
.8
.6
.4
.2
0
ARBITRARY Y
d
b
a
c
860
880
900
920
940
960
980
RAMAN SHIFT (cm-1)

FIG. 5

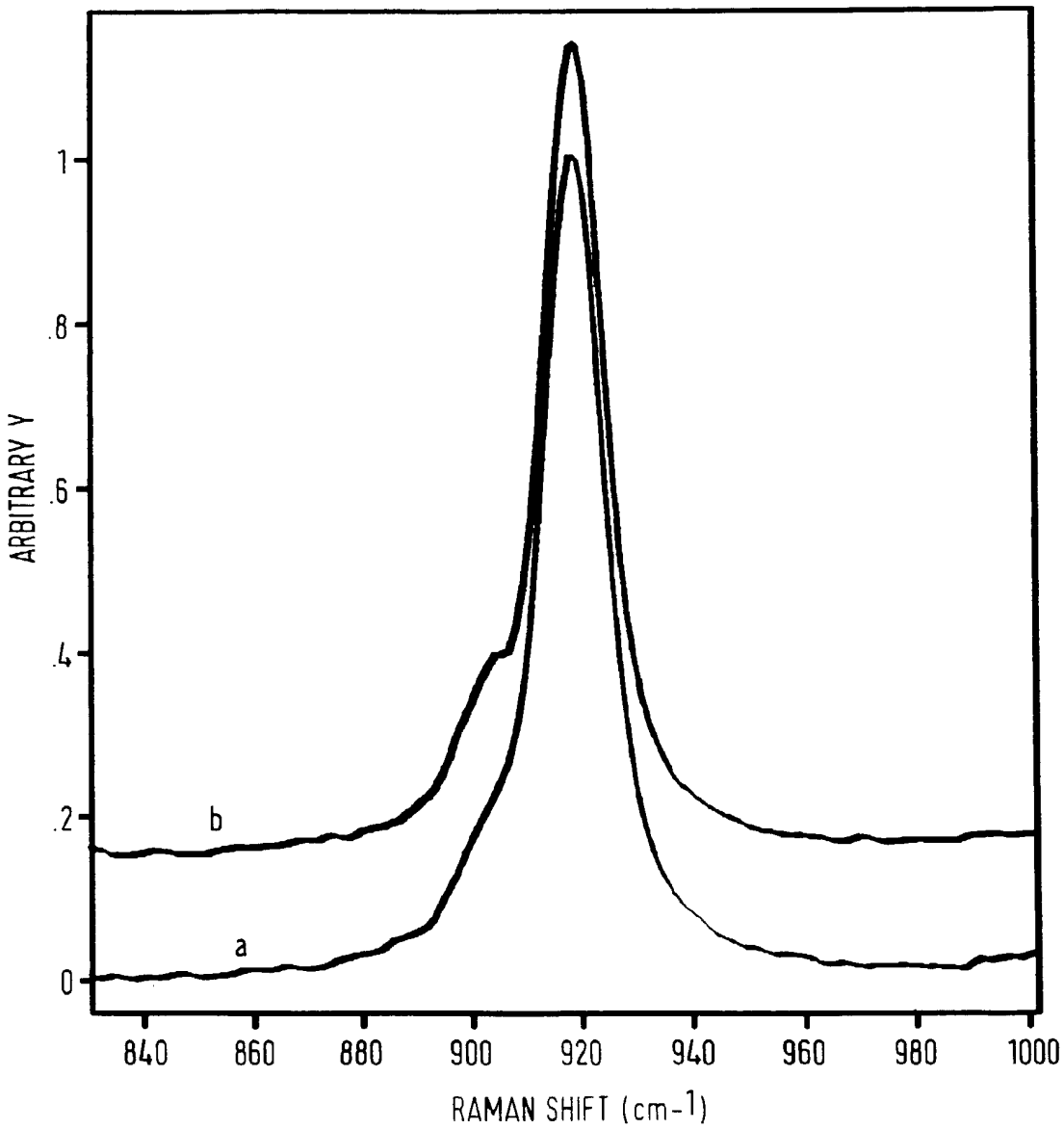

ARBITRARY Y
1
.8
.6
.4
.2
0
b
a
840
860
880
900
920
940
960
980
1000
RAMAN SHIFT (cm-1)

FIG. 6

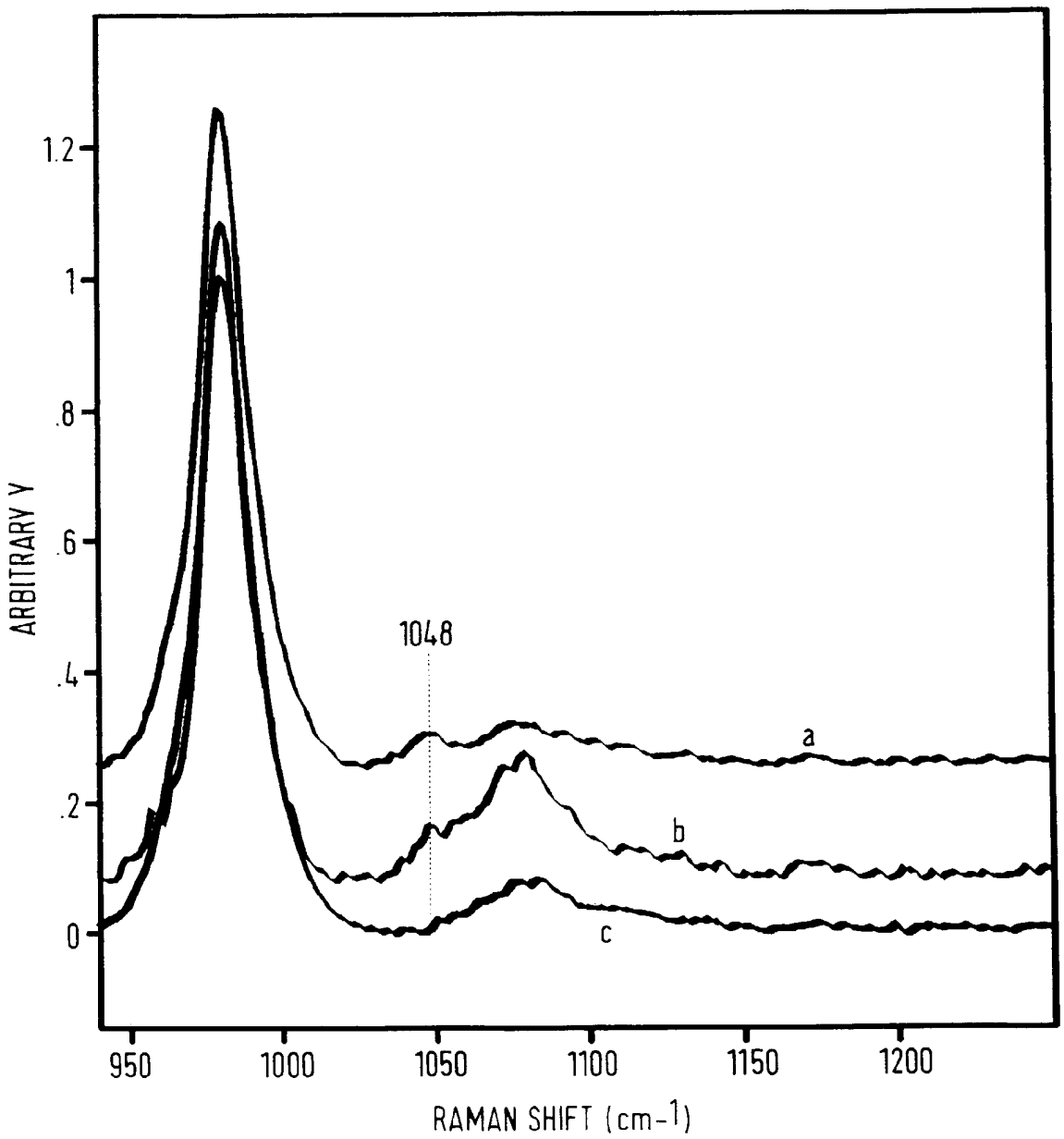

1.2
1
.8
.6
.4
.2
0
ARBITRARY Y
1048
a
b
c
950
1000
1050
1100
1150
1200
RAMAN SHIFT (cm-1)

FIG. 7

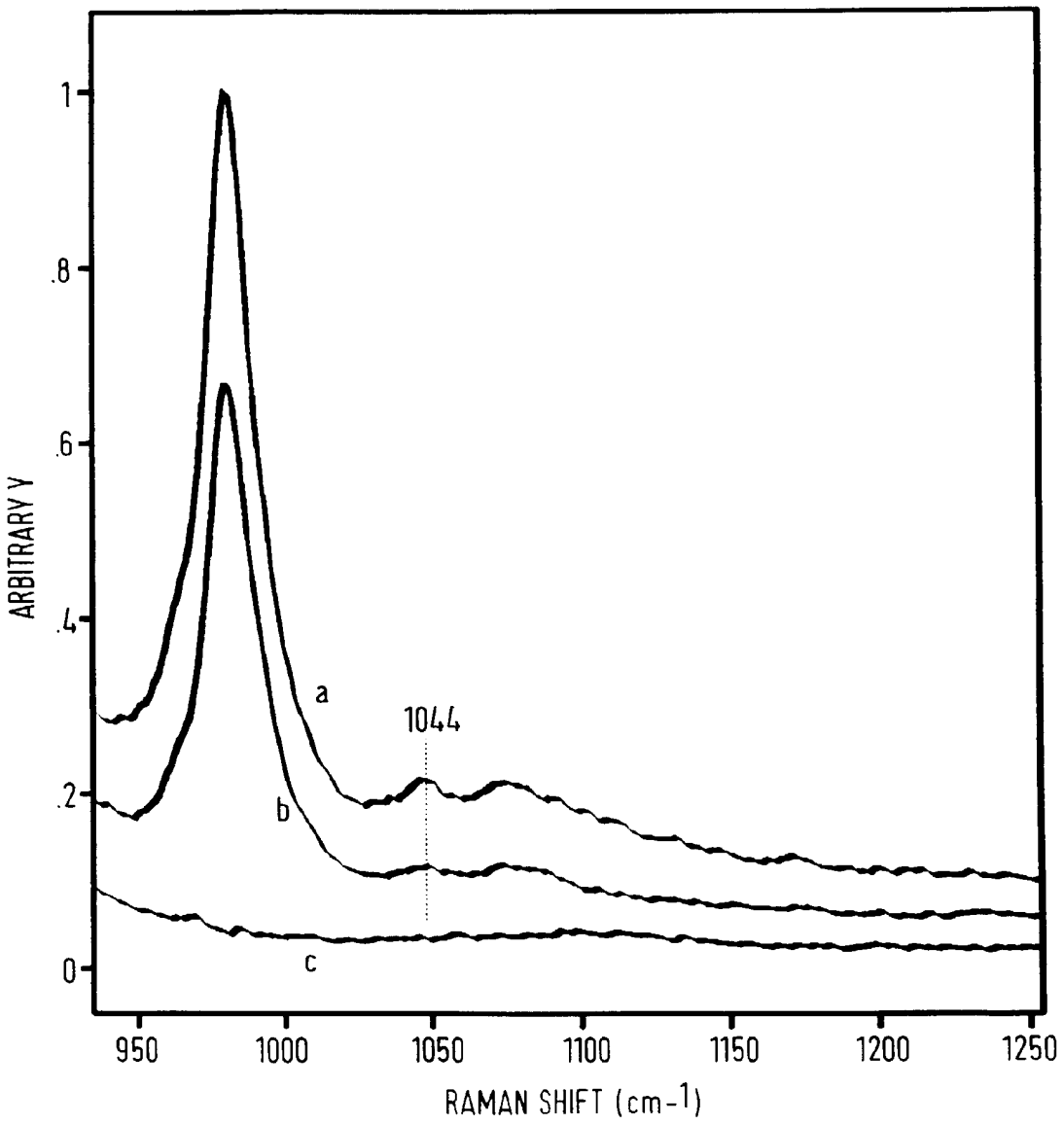

1
.8
.6
.4
.2
0
ARBITRARY Y
a
b
c
1044
950
1000
1050
1100
1150
1200
1250
RAMAN SHIFT (cm-1)

FIG. 8

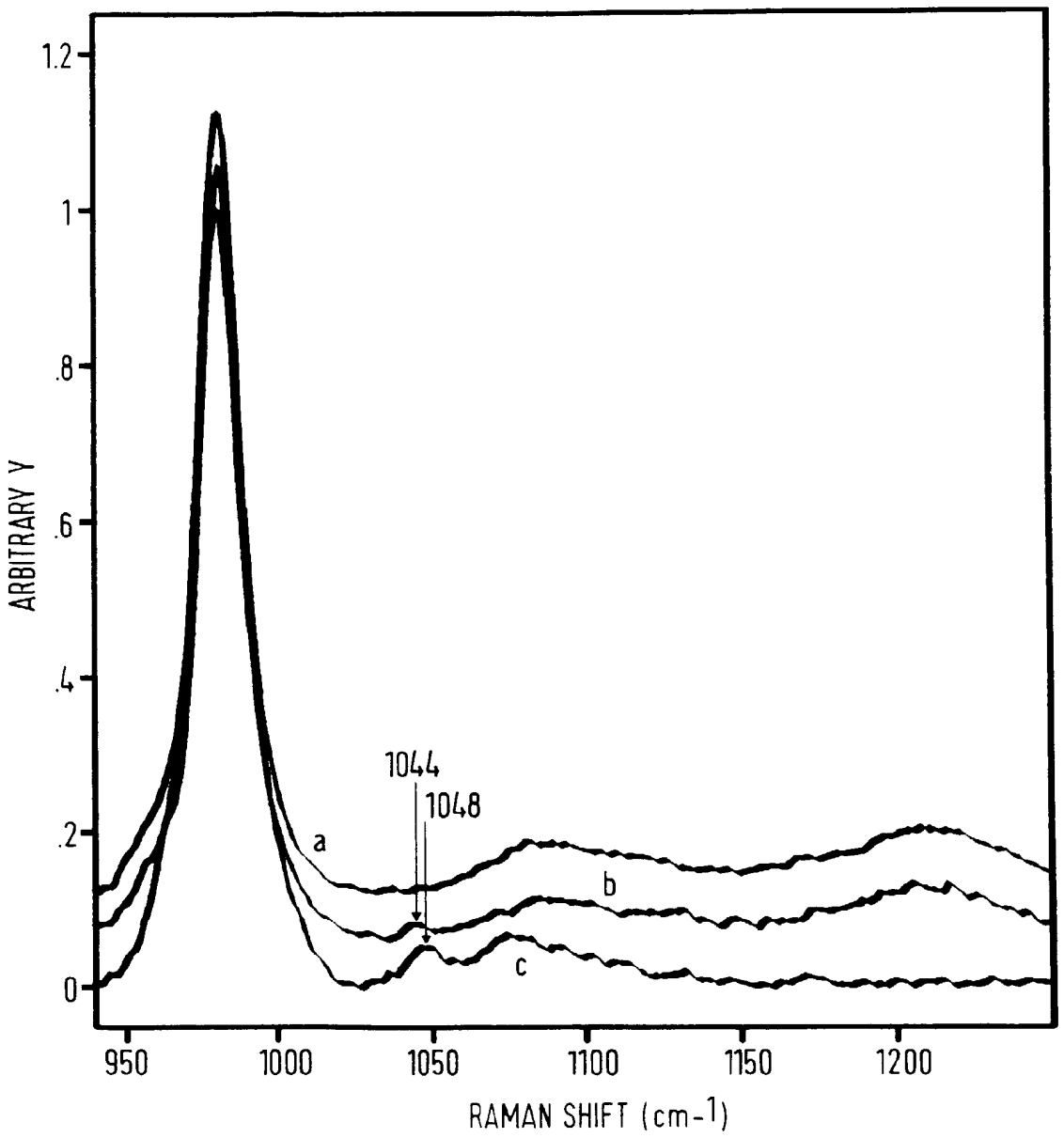

1.2
1
.8
.6
.4
.2
0
ARBITRARY Y
1044
1048
a
b
c
950
1000
1050
1100
1150
1200
RAMAN SHIFT (cm-1)

FIG. 9

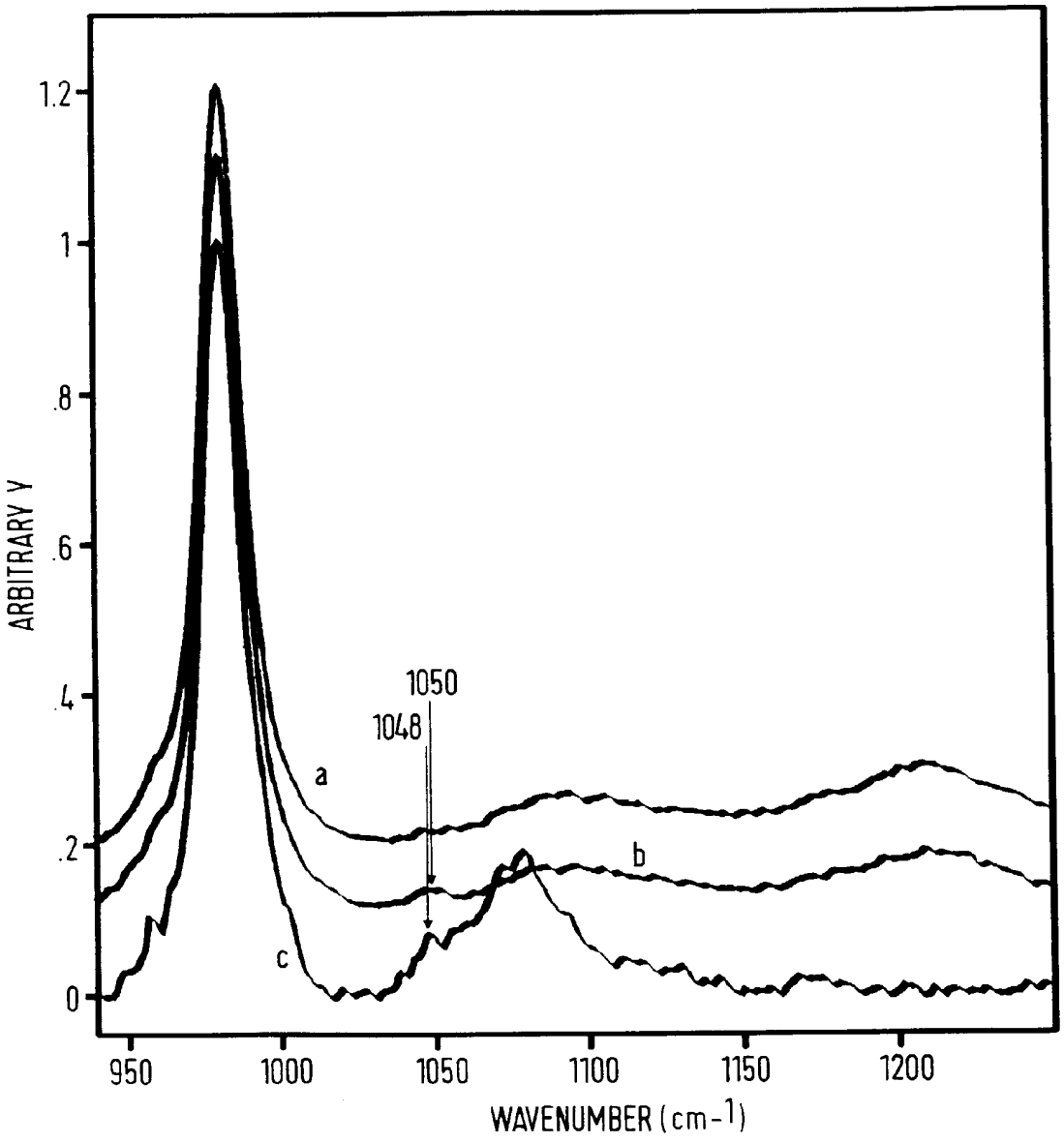

1.2
1
.8
.6
.4
.2
0
ARBITRARY Y
1050
1048
a
b
c
950
1000
1050
1100
1150
1200
WAVENUMBER (cm-1)

FIG. 10

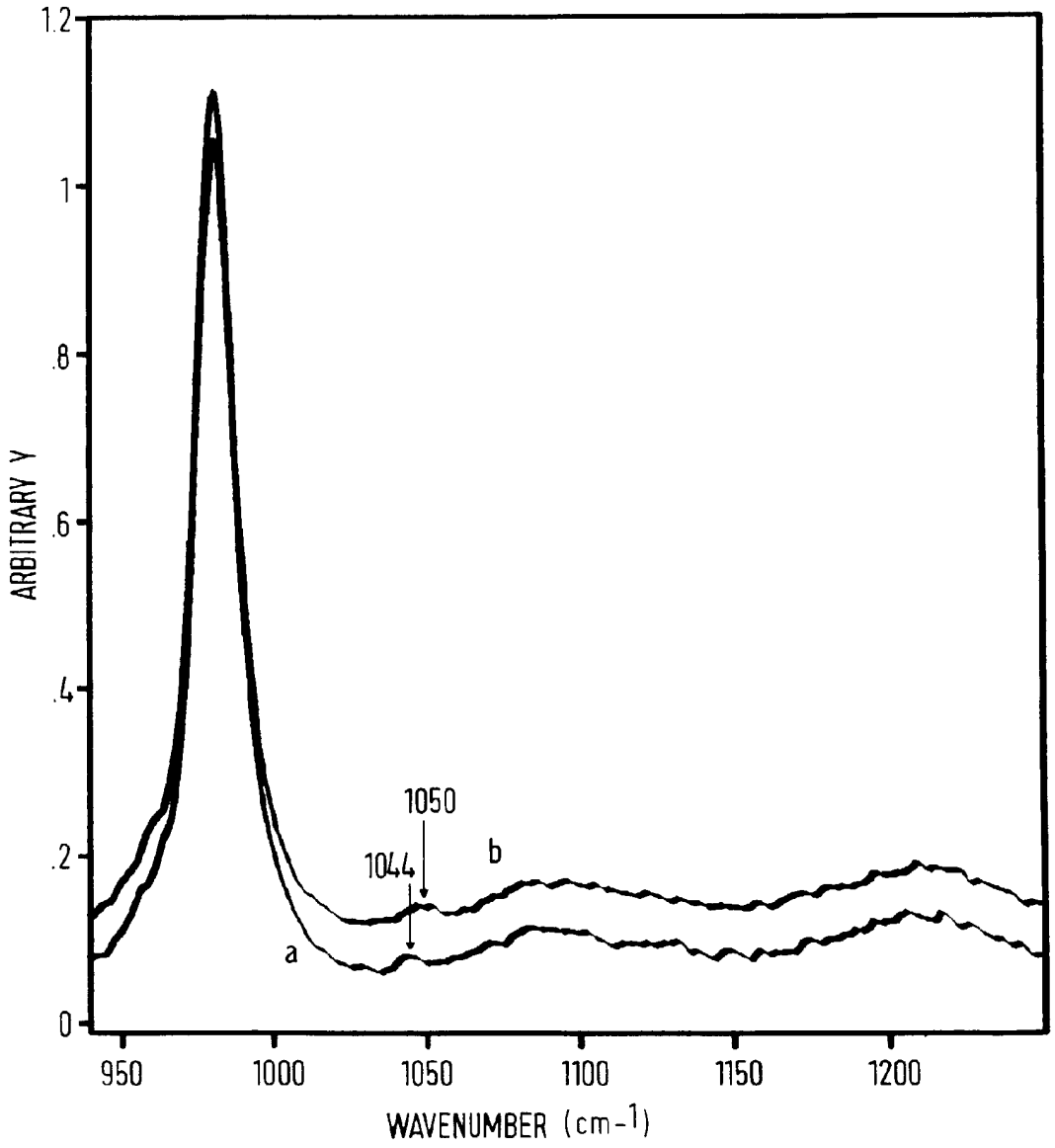

1.2
1
.8
.6
.4
.2
0
ARBITRARY Y
1050
1044
b
a
950
1000
1050
1100
1150
1200
WAVENUMBER (cm-1)

FIG. 11

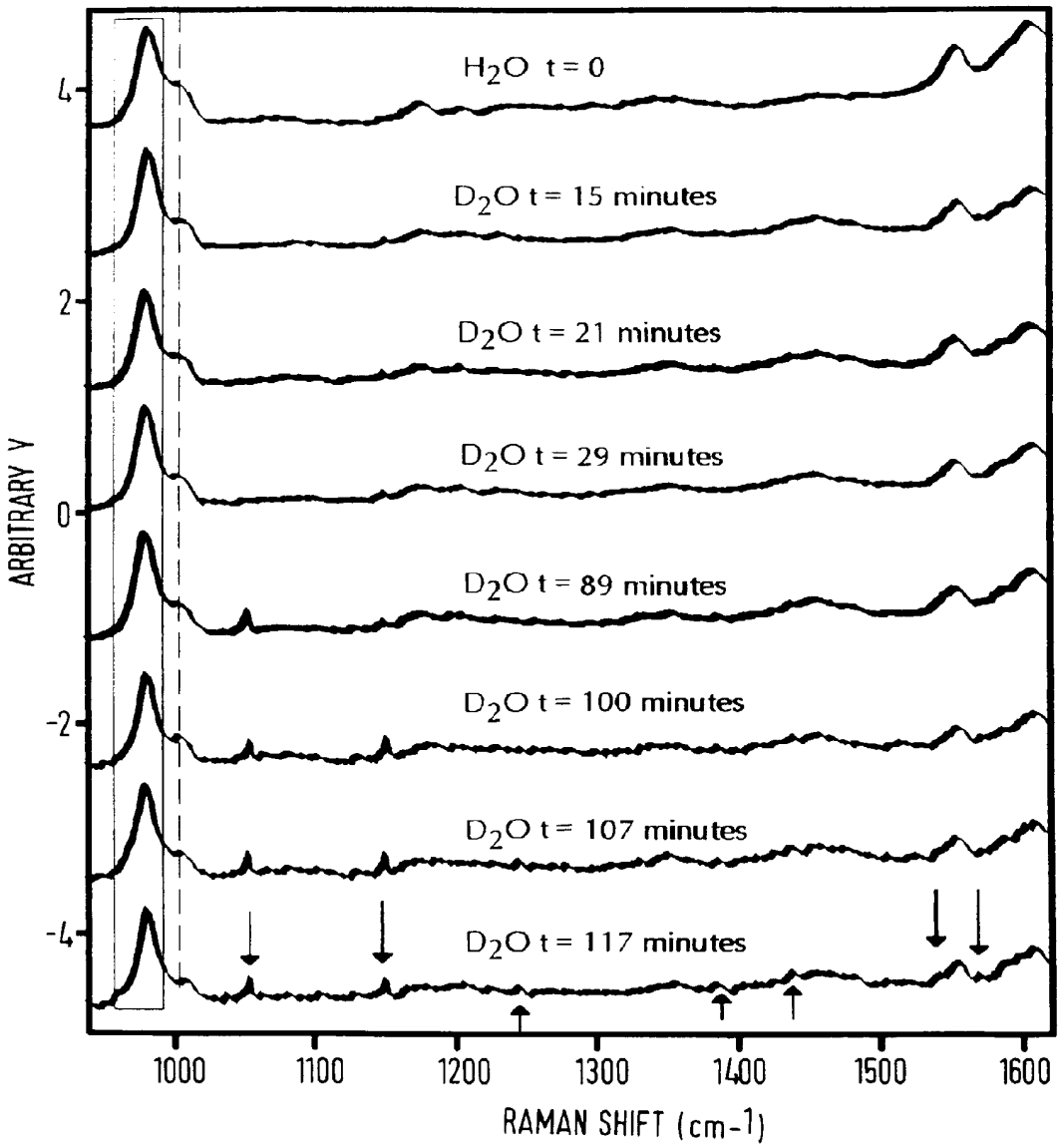

H2O t = 0
D2O t = 15 minutes
D2O t = 21 minutes
D2O t = 29 minutes
D2O t = 89 minutes
D2O t = 100 minutes
D2O t = 107 minutes
D2O t = 117 minutes
ARBITRARY Y
4
2
0
-2
-4
1000
1100
1200
1300
1400
1500
1600
RAMAN SHIFT (cm-1)

FIG. 12

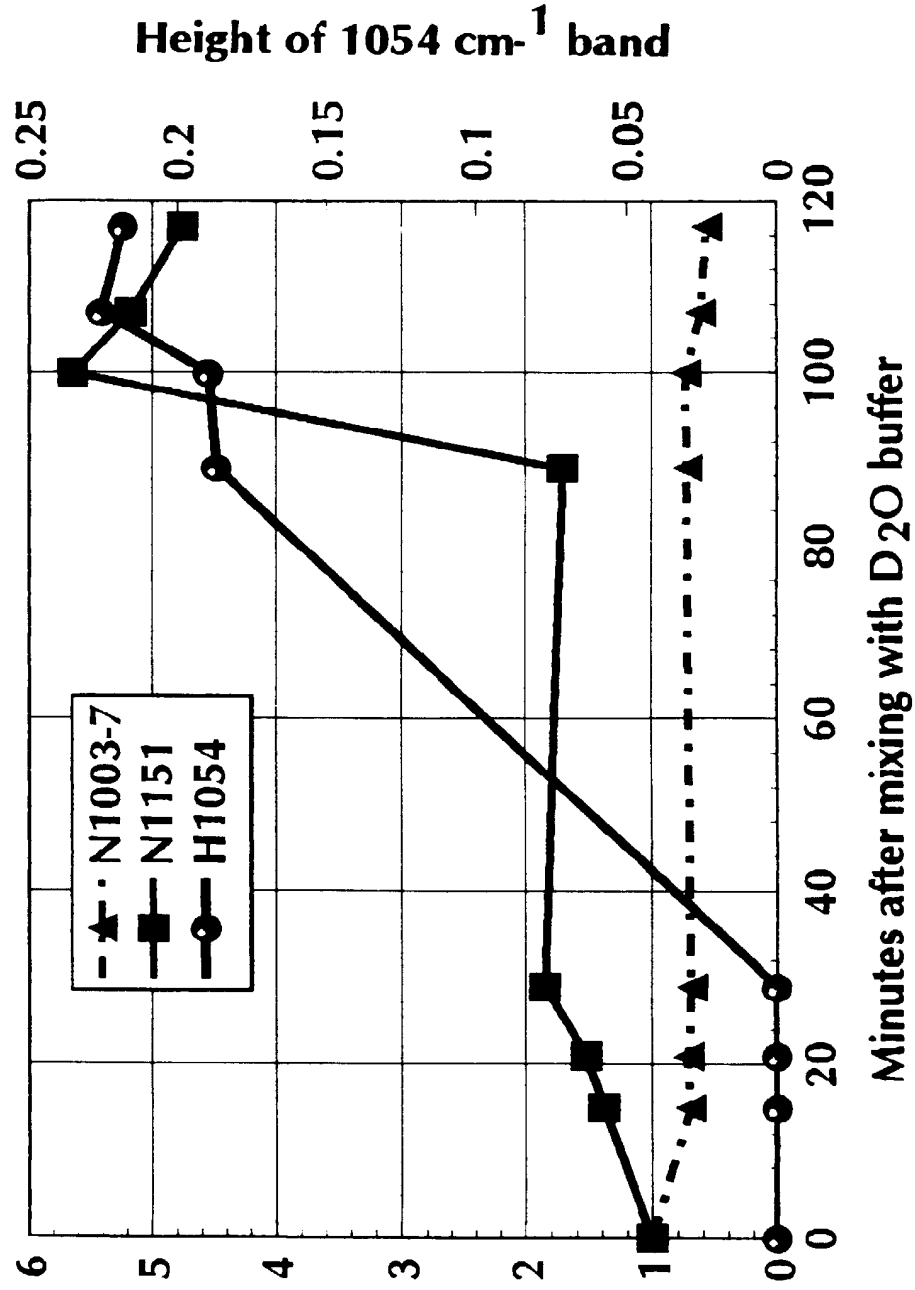

Height of 1054 cm-1 band
0.25
0.2
0.15
0.1
0.05
0
120
100
80
60
40
20
0
Minutes after mixing with D2O buffer
N1003-7
N1151
H1054
6
5
4
3
2
1
0
Intensities of 1003-7 and 1151 cm-1 bands,
Normalized to t=0 minutes values
in H2O buffer

FIG. 13

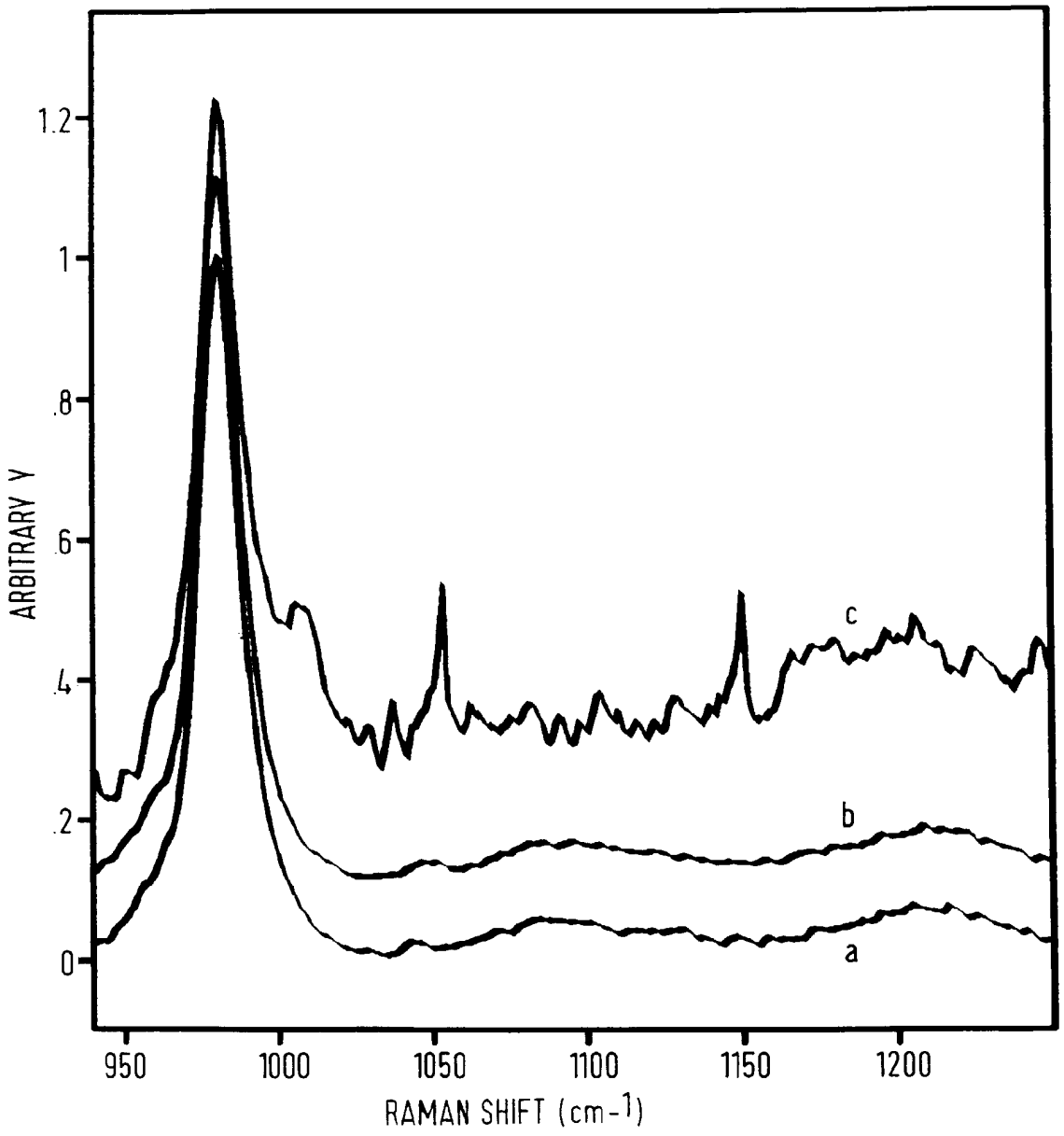

1.2
1
.8
.6
.4
.2
0
ARBITRARY Y
950
1000
1050
1100
1150
1200
RAMAN SHIFT (cm-1)
a
b
c

FIG. 14

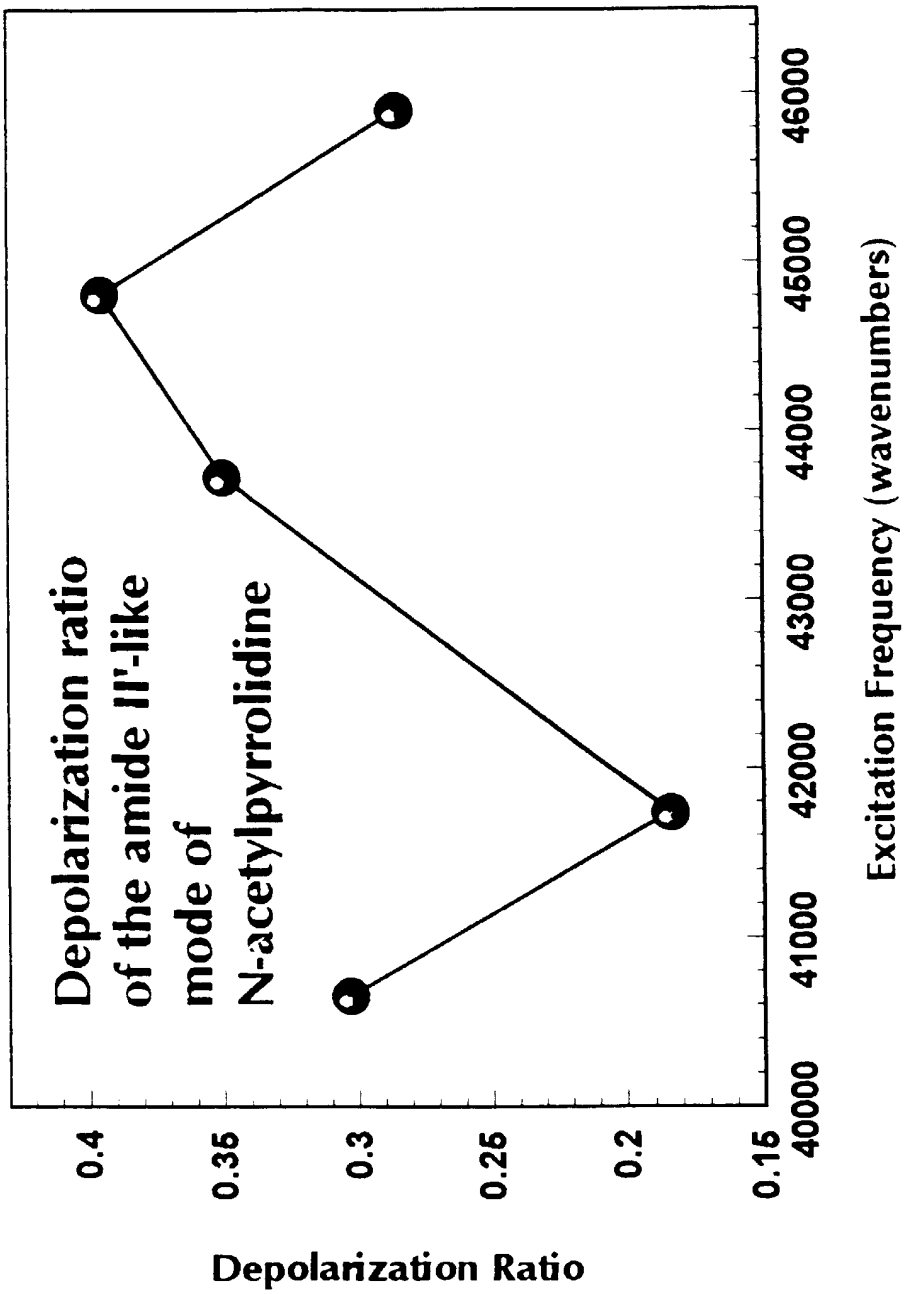

Depolarization ratio of the amide II'-like mode of N-acetylpyrrolidine
Depolarization Ratio
0.4
0.35
0.3
0.25
0.2
0.15
40000
41000
42000
43000
44000
45000
46000
Excitation Frequency (wavenumbers)

FIG. 15

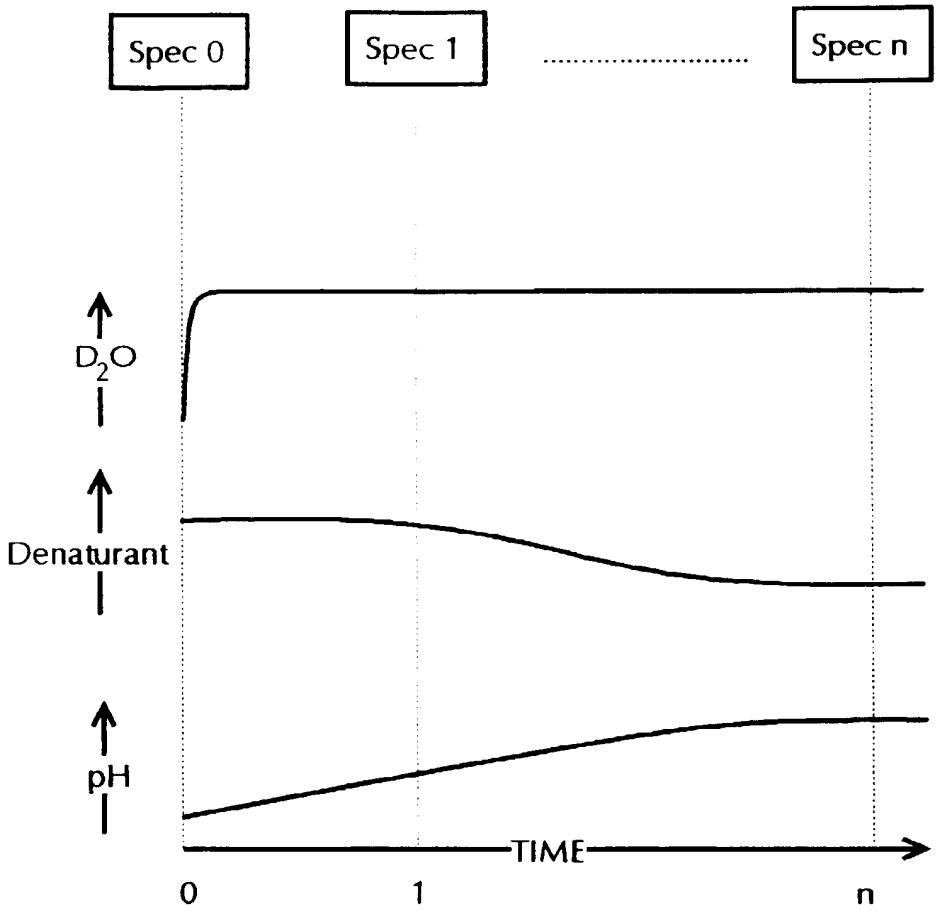
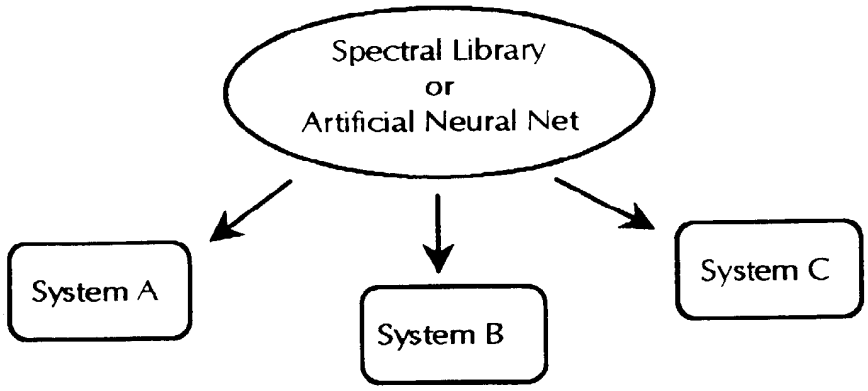


FIG. 16

FIG. 17

A - With Depolarizer



B - Without Depolarizer